

# **Nitric and Nitrous Oxide Emission from an Upland Agricultural Grassland Soil**

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## Declaration

I am responsible for composing this thesis. It represents my own work and where the work of others has been used it is duly acknowledged.

Signed.

Date.....27/07/04.....

## Abstract

Nitric and nitrous oxide are products of the microbial processes of nitrification and denitrification in soils; they are both important gases in that they contribute to stratospheric ozone depletion and climate change. The effects of land management as fertiliser and lime addition on the function and microorganisms contributing to nitrification and denitrification is poorly understood. It is therefore important to understand the factors affecting both the microbial processes themselves and in turn how this influences their activity in the field.

Soil microcosm experiments were used to quantify the effects of changing soil moisture and temperature as an environmental factor on the dominant production of nitric and nitrous oxide in soil sampled from an un-improved upland grassland field in the Scottish borders (Sourhope). Microcosms were amended with  $\text{NH}_4\text{NO}_3$  at the field application rate of  $126 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , nitric oxide flux increased significantly at soil moisture contents below 60% WFPS (Water Filled Pore Space), the maximum flux was  $0.134 \mu\text{g NO} - \text{N g}^{-1} \text{ h}^{-1}$  at 40% WFPS. Nitrous oxide flux however, only increased at 80% WFPS with a flux of  $9.942 \times 10^{-3} \mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{ h}^{-1}$  and decreased over a period of 27 days to  $2.09 \times 10^{-6} \mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{ h}^{-1}$ , similar to that of the other soil WFPS microcosms.

The effect of changing soil temperature on nitric and nitrous oxide emissions at 40 and 80% WFPS and 1,5,10,15,20 and 25 °C were quantified. At low soil moisture contents the maximum flux of nitric and nitrous oxide,  $0.17 \mu\text{g NO-N g soil}^{-1} \text{ h}^{-1}$  and  $4.19 \times 10^{-5} \mu\text{g N}_2\text{O-N g soil}^{-1} \text{ h}^{-1}$  respectively, was at 15°C. The flux of both decreased with increasing temperature at 40% WFPS.

A collaborative long term incubation study using soil microcosms with soil sampled from the control treatments at Sourhope was set up to quantify the relationship between function and diversity. This study showed that land management regimes in upland agricultural grassland soils as inorganic N fertiliser addition and lime, may result in increased N loss as nitric and nitrous oxide via both nitrification and denitrification at 60% WFPS. Increases in both nitric and nitrous

oxide emissions could not be correlated with increases in gross nitrification rates due to the large variation within treatments, however the proportion of nitrous oxide produced via nitrification was greater than that produced via denitrification for all treatments. There were shifts in the number of bands for all three functional groups measured using DGGE 16sRNA, although changes in the eubacterial DGGE profiles were greater indicating that both the AMO and *Nitrobacter sp.* ammonium and nitrite oxidisers were relatively stable. Following  $^{15}\text{N}$  analyses of the nitrous oxide and  $\text{N}_2$  produced was predominantly via nitrification with no  $^{15}\text{N}$  labelled  $\text{N}_2$  produced indicating that nitrification was the predominant source of nitrous oxide.

Inhibition work in re-packed soil microcosms with soil from the Sourhope field treatments was carried out using DCD (an autotrophic inhibitor) and cyclohexamide (a heterotrophic inhibitor). The results from these studies showed a reduction in the nitrous oxide emission in lime amended soils relative to control soils without inhibitor, by 37% in the lime only and 80% in the nitrogen and lime treatments. Since the  $^{15}\text{N}$  labelling of the nitrous oxide produced indicated that more than 99% was due to nitrification it would seem that decreased emissions as a result of liming are due to changes in the nitrifier rather than denitrifier populations.

Field flux measurements were made throughout 2000/2001 with a period of reduced activity due to the foot and mouth epidemic in the UK during 2001. Field measurements have shown that nitric and nitrous emissions and soil mineral N concentrations in the four week period following the addition of ammonium nitrate were significantly greater than those in control plots. Emission of nitric and nitrous oxide were further increased to  $0.26 \mu\text{g NO-N m}^{-2} \text{ h}^{-1}$  and  $0.4 \mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$  respectively by addition of nitrogen combined with lime. Control soils and soils treated with lime only acted as a net sink for nitrous oxide. Following stepwise multiple regression analyses rainfall, air temperature and soil ammonium concentrations were important factors for modelling the emissions of nitrous oxide whereas soil WFPS and soil nitrate concentrations were important for nitric oxide emissions, these findings are in agreement with regression equations in the DNDC model.



## Aims

- Quantify the effects of land management regimes on the nitric and nitrous oxide flux in the field and measure changes in soil inorganic ammonium and nitrate pools over two field seasons. The effects of *in situ* soil temperature and moisture were also determined in the field to try and quantify dominant processes for the flux of nitric and nitrous oxide following lab microcosm studies. Diurnal field flux measurements were also made to validate overall nitric and nitrous oxide fluxes in the field.
- Lab microcosm studies were used to determine the effects of soil temperature and moisture on the nitric and nitrous oxide flux under controlled conditions. Specific inhibitors of autotrophic and heterotrophic nitrification were also used to quantify the relative contribution of these two pathways for nitric and nitrous oxide flux as a result of land management in soil microcosms.
- Longer term enrichment studies aimed to measure changes in function as nitric and nitrous oxide flux and gross nitrification as a direct result of land management treatment applications. This was closely linked to molecular work and  $^{13}\text{C}$  labelled,  $\text{CO}_2$  pulse labelling of microbial DNA and RNA (Results not presented in this thesis) carried out as part of this Soil Biodiversity NERC thematic programme .
- These data will contribute to the growing understanding of the complex processes involved in the nitrogen cycle in soils and provide data for use in models of N cycling and biodiversity.

## Contents

<b>Chapter One. Introduction.....</b>	<b>1</b>
1.1. The global nitrogen cycle.....	1
1.1.1. Di-Nitrogen fixation.....	2
1.1.2. Organic nitrogen mineralisation.....	2
1.1.3. Nitrification .....	4
1.1.4. Denitrification .....	4
1.1.5. Leaching.....	5
1.2. NO and N <sub>2</sub> O atmospheric budget (sources sinks and sizes, acid rain) .....	5
1.2.1. Nitric oxide .....	6
1.2.2. Nitrous oxide.....	7
1.3. NO <sub>2</sub> <sup>-</sup> , NO <sub>3</sub> <sup>-</sup> and NH <sub>4</sub> <sup>+</sup> terrestrial budget .....	9
1.4. Importance of biodiversity on the soil nitrogen cycle.....	9
1.4.1. Quantification of microbial biodiversity .....	12
1.5. Importance of nitrogen cycling to global climate change.....	12
1.5.1. IPCC / Kyoto protocol / current state of affairs .....	12
1.5.2. Radiative forcing by oxides of nitrogen.....	13
1.6. Production and consumption of NO and N <sub>2</sub> O in soils .....	15
1.6.1. Nitrification (autotrophic/heterotrophic).....	15
1.6.2. Denitrification (autotrophic/heterotrophic).....	17
1.6.3. Dissimilatory/assimilatory nitrate reduction.....	19
1.6.4. Chemodenitrification .....	20
1.7. Factors affecting NO and N <sub>2</sub> O emission and production in soils .....	20
1.7.1. Soil water content (WFPS).....	20

1.7.2.	Nitrogen availability (type and source).....	22
1.7.3.	Soil pH (liming) .....	23
1.7.4.	Soil and air temperature .....	24
1.7.5.	Microbial species diversity .....	24
1.7.6.	Carbon availability (type and source) .....	25
1.8.	The use of stable isotopes .....	25
1.8.1.	Measurement of $\delta^{15}\text{N}$ .....	26
1.8.2.	Factors affecting $\delta^{15}\text{N}$ isotope ratios in the nitrogen cycle .....	26
1.8.3.	$^{15}\text{N}$ pool dilution and enrichment.....	28
1.8.4.	Natural abundance $^{15}\text{N}$ .....	29
<b>Chapter Two. General Methods .....</b>		<b>32</b>
2.1.	Field site description .....	32
2.2.	The measurement of trace gas fluxes .....	36
2.3.	Chamber design.....	36
2.4.	Methods of analysis of nitric oxides .....	38
2.5.	Methods of analysis of nitrous oxide .....	40
2.6.	The measurement of trace gas fluxes in soil microcosm experiments.....	43
2.7.	Measurement of nitrous oxide.....	46
2.8.	Soil inorganic nitrogen analysis .....	47
2.9.	$^{15}\text{N}$ stable isotope pool dilution.....	48
<b>Chapter Three. Method Development and Evaluation .....</b>		<b>51</b>
3.1.	Evaluation of $\text{NO}_x$ reactions within a PTFE chamber .....	51
3.2.	Microcosm dynamic NO flux system calibration .....	54
3.3.	Nitrite detection and extraction from soil .....	57



3.4. Quantification of nitric oxide sources following nitrite addition to sterile soil .....	58
3.5. Effects of soil weight on NO and N <sub>2</sub> O flux in soil microcosms .....	60
3.6. The effects of sweep air flow rate on NO flux in soil microcosms.....	62
<b>Chapter Four. Effects of Soil temperature and moisture content on nitric and nitrous oxide emission in soil microcosms .....</b>	<b>66</b>
4.1. Introduction .....	66
4.1.1. Effects of soil moisture content.....	67
4.1.2. Effects of soil temperature .....	68
4.2. Materials and Methods .....	69
4.2.1. Soil sampling and field site .....	69
4.2.2. Soil moisture experiment .....	69
4.2.3. Soil temperature experiment .....	70
4.2.4. Gaseous N determination and inorganic N analyses.....	71
4.3. Results .....	71
4.3.1. Effects of soil moisture content .....	71
4.3.2. Effects of soil temperature .....	78
4.4. Discussion .....	82
4.4.1. Soil moisture .....	82
4.4.2. Soil temperature .....	84
<b>Chapter Five. Effects of specific inhibitors on soil microcosm nitric and nitrous oxide flux.....</b>	<b>87</b>
5.1. Introduction .....	87
5.1.1. Specific inhibitors of nitrification .....	87
5.2. Materials and methods .....	89



5.2.1 Soils and sampling .....	89
5.2.2. Treatment structure .....	89
5.2.3. Nitrification rates .....	90
5.3. Results .....	91
5.3.1. Nitric and nitrous oxide flux inhibition.....	91
5.3.2. Effects of specific inhibitors on gross rates of nitrification and <sup>15</sup> N – N <sub>2</sub> O enrichment .....	94
5.4. Discussion .....	96
<b>Chapter Six. Long term incubation of soil microcosms under different land management regimes. ....</b>	<b>100</b>
6.1. Introduction .....	100
6.1.1. Land management and nitrogen cycle.....	100
6.2. Materials and Methods.....	102
6.2.1. Experimental set-up .....	102
6.2.2. Microcosm CO <sub>2</sub> labeling.....	107
6.2.3. Nitric and nitrous oxide flux measurements .....	108
6.2.4. Nitrification and <sup>15</sup> N pool dilution .....	109
6.2.5. Molecular sampling and methods .....	109
6.3. Results .....	110
6.3.1. NO and N <sub>2</sub> O flux.....	110
6.3.3. <sup>15</sup> N pool dilution of microcosms incubated with Sourhope treatments for 4 months. Gross nitrification rates and <sup>15</sup> N - N <sub>2</sub> O data. ....	113
6.3.4. Molecular results.....	121
6.4. Discussion .....	126

<b>Chapter Seven. Field flux measurements of nitric and nitrous oxide.</b>	<b>133</b>
7.1. Introduction	133
7.2. Materials and Methods	136
7.2.1. Soil and vegetation	136
7.2.2. Gaseous N measurements	136
7.2.3. Soil sampling and inorganic N	137
7.2.4. Diurnal field flux measurements	137
7.3. Results	138
7.3.1. Soil moisture and temperature	138
7.3.2. Nitric and nitrous oxide flux	140
7.3.3. Soil inorganic nitrogen	142
7.3.4. Diurnal nitric and nitrous oxide flux	148
7.4. Discussion	150
<b>Chapter Eight. General Discussion</b>	<b>155</b>
8.1. Main findings	155
8.2. Effects of environmental conditions on nitric and nitrous oxide emissions	157
8.4. Effects of nitrogen and lime on autotrophic and heterotrophic nitrification	161
8.5. Soil long term incubation and microbial diversity and function	163
8.6. Effects of land management on the emissions of nitric and nitrous oxide in the field	165
<b>References</b>	<b>168</b>
<b>Appendix 1</b>	<b>184</b>
<b>Appendix 2</b>	<b>188</b>

## List of Figures

Figure 1. Nitrogen cycle.....	1
Figure 2. Global sources of N <sub>2</sub> O (Tg N yr <sup>-1</sup> ) (IPCC, 1994).....	8
Figure 3. Atmospheric chemistry of nitric oxide and nitrogen dioxide with ozone. .	14
Figure 4. Production of nitric acid via complex interconversion sequences.....	14
Figure 5. Stratospheric sinks for nitrous oxide .....	14
Figure 6. Model of nitrogen flux as proposed by Firestone and Davidson (1989)....	15
Figure 7. The autotrophic nitrification pathway .....	16
Figure 8. The heterotrophic nitrification pathway .....	16
Figure 9. The denitrification pathway .....	18
Figure 10. DNRA reaction sequence .....	20
Figure 11. Summary of $\delta^{15}\text{N}$ measurements of various nitrogen species (Trumbore, 1995 and Macko & Ostrom, 1994) .....	30
Figure 12. Sourhope randomised block treatment allocations .....	34
Figure 13. Sourhope sub-plot design .....	35
Figure 14. Static non-vented chamber used for field gas measurements .....	38
Figure 15. Schematic diagram of Chrompak GC and backflush valve system.....	42
Figure 16. Soil microcosm design.....	43
Figure 17. Schematic diagram of NO flux measurement system for soil microcosm experiments, arrows indicate the flow of lab air. ....	44
Figure 18. Aluminium block for making PTFE acid traps .....	49
Figure 19. <sup>15</sup> N extraction method.....	50
Figure 20. Ambient ozone concentration measured at the CEH Edinburgh Bush Estate constant monitoring station for the two PTFE chamber measurement days. ....	51



Figure 21. NO reactions with ozone and chamber walls within a PTFE chamber at low ambient ozone concentrations (09/10/00).	52
Figure 22. NO reactions with ozone and chamber walls within a PTFE chamber at high ambient ozone concentrations (05/06/02).	53
Figure 23. Simple linear regression of data from the 0-100 sccm MFC.	56
Figure 24. Simple linear regression of data for the 0-2.5 slpm MFC	56
Figure 25. Nitric oxide production following nitrite addition to soil microcosms at 40% WFPS in sterile and non sterile soil microcosms (Act = acetate, nitrite = $\text{NaNO}_2$ ).	59
Figure 26. The effects of soil weight on nitric oxide flux calculations (a) and mixing	61
Figure 27. Linear relationship between log transformed nitric oxide flux and soil dry weight (n=2 for each soil dry weight)	62
Figure 28. The effects of sweep air flow rate on NO flux in $\mu\text{g g}^{-1} \text{h}^{-1}$ and NO mixing ratios	64
Figure 29. Model of nitric and nitrous oxide production as a function of WFPS from Davidson (1991).	68
Figure 30. Effects of soil moisture content on the nitrous (a) and nitric oxide (b) flux in soil microcosms incubated at 15°C following addition of $\text{NH}_4\text{NO}_3$ (error bars $\pm$ S.E.)	72
Figure 31. Simple linear regression between soil moisture content and nitric oxide flux in soil microcosms incubated at 15°C 2 days post $\text{NH}_4\text{NO}_3$ addition ( $r^2 = -0.88$ , $p < 0.001$ )	73
Figure 32. Effects of soil moisture content on soil nitrate (a) and ammonium (b)....	74
Figure 33. Average nitric and nitrous oxide fluxes and soil exchangeable nitrate concentrations as a function of incubation time a) and b) and soil moisture content c) and d) following $\text{NH}_4\text{NO}_3$ addition to soil microcosms incubated at 15°C.	76



- Figure 34. Average nitric and nitrous oxide fluxes and soil exchangeable ammonium concentrations as a function of incubation time a) and b) and soil moisture content c) and d) following  $\text{NH}_4\text{NO}_3$  addition to soil microcosms incubated at  $15^\circ\text{C}$ . ..... 77
- Figure 35. Effects of soil temperature on nitric and nitrous oxide flux at 40 (a) and (b) and 80% (c) and (d) WFPS in soil microcosms following addition of  $\text{NH}_4\text{NO}_3$ . ..... 78
- Figure 36. Effects of soil temperature on soil microcosm NO flux and NO mixing ratios at 40 and 80% WFPS on day 1 (a) and day 2 (b) following addition of  $\text{NH}_4\text{NO}_3$ . (error bars = SE,  $n = 3$  for both x and y data) ..... 80
- Figure 37. Effects of soil WFPS on nitric and nitrous oxide flux following  $\text{NH}_4\text{NO}_3$  addition to soil microcosms at  $15^\circ\text{C}$  ..... 83
- Figure 38. The effects of DCD and cyclohexamide on nitric and nitrous oxide flux in soil microcosms. Nitrous oxide flux was measured on day one following treatment and inhibitor additions. The soil exchangeable N results are presented from soil microcosms extracted at the end of the microcosm incubation after 14 days (C – control; N –  $\text{NH}_4\text{NO}_3$ ; L – Lime; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; Aut – Beachgrove soil; Cyc – cyclohexamide; DCD – dicyandiamide)..... 93
- Figure 39. Changes in soil nitrate concentration and  $^{15}\text{N}$  enrichment following pool dilution at incubation  $t_{3d}$  (a) and  $t_{0d}$  (b) after two weeks of microcosm inhibition. The effects of inhibitor on the  $^{15}\text{N}$  enrichment of nitrous oxide (c) and gross nitrification rates determined from pool dilution equations (d) (C – Control; L – Lime; N –  $\text{NH}_4\text{NO}_3$ ; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; Cyc – cyclohexamide; DCD – Dicyandiamide) ..... 95
- Figure 40. Schematic diagram of long term enrichment microcosm set-up. .... 106
- Figure 41. Nitric and nitrous oxide flux in soil microcosms following addition of treatments at 1 and 21 wk field equivalents a-b and c-d respectively. The effects of  $\text{NH}_4\text{NO}_3$  addition on nitric and nitrous oxide flux following 6 months of long term incubation e-f (C-1 – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –

NH <sub>4</sub> NO <sub>3</sub> + Acetate; N + L - NH <sub>4</sub> NO <sub>3</sub> + Lime; N + L + Act - NH <sub>4</sub> NO <sub>3</sub> + Lime + Acetate; Nit - NaNO <sub>2</sub> ; Nit + Act - NaNO <sub>2</sub> + Acetate).....	111
Figure 42. Gross nitrification rates in soil microcosms following <sup>15</sup> N pool dilution. (C-1 – control; L – Lime (CaCO <sub>3</sub> ); N – NH <sub>4</sub> NO <sub>3</sub> ; N + Act – NH <sub>4</sub> NO <sub>3</sub> + Acetate; N + L - NH <sub>4</sub> NO <sub>3</sub> + Lime; N + L + Act - NH <sub>4</sub> NO <sub>3</sub> + Lime + Acetate; Nit – NaNO <sub>2</sub> ; Nit + Act – NaNO <sub>2</sub> + Acetate). (Error bars are ± S.E. n=3) .....	
	114
Figure 43. Isotopic composition of nitrous oxide produced following addition of <sup>15</sup> N labelled NO <sub>3</sub> <sup>-</sup> (99 atom % excess) as NH <sub>4</sub> NO <sub>3</sub> to soil microcosms after 6 months of treatment enrichment (C-1 – control; L – Lime (CaCO <sub>3</sub> ); N – NH <sub>4</sub> NO <sub>3</sub> ; N + Act – NH <sub>4</sub> NO <sub>3</sub> + Acetate; N + L - NH <sub>4</sub> NO <sub>3</sub> + Lime; N + L + Act - NH <sub>4</sub> NO <sub>3</sub> + Lime + Acetate; Nit – NaNO <sub>2</sub> ; Nit + Act – NaNO <sub>2</sub> + Acetate).....	
	115
Figure 44. Contribution of nitrification and denitrification to N <sub>2</sub> O production following <sup>15</sup> N pool dilution in long term incubation soil microcosms (C – Control; L – Lime; N – NH <sub>4</sub> NO <sub>3</sub> ; N + Act – NH <sub>4</sub> NO <sub>3</sub> + Acetate; N + L – NH <sub>4</sub> NO <sub>3</sub> + Lime; N + L + Act – NH <sub>4</sub> NO <sub>3</sub> + Lime + Acetate; Nit – NaNO <sub>2</sub> ; Nit + Act – NaNO <sub>2</sub> + Acetate).....	
	117
Figure 45a. Linear regression between nitrous oxide and nitrate reduction to nitrous oxide fractionation factors (α) for all treatments. ....	
	118
Figure 45b. Linear regression between nitrous oxide and ammonium reduction to nitrous oxide fractionation factors (α) for all treatments.....	
	118
Figure 46. Effects of long term treatment incubations on nitric and nitrous oxide flux (a) (linear regression for data points other than lime treatments, R <sup>2</sup> = 1, p <0.001); relationship between nitrous oxide flux and <sup>15</sup> N enrichment of nitrous oxide (b) and gross nitrification rates and nitric to nitrous oxide ratios (c).....	
	120
Figure 47 a. DGGE analysis of 16S rRNA genes amplified from extracted DNA using eubacteria, primers. C – control, N – NH <sub>4</sub> NO <sub>3</sub> , L – lime, Ac – acetate, T <sub>0</sub> – initial value, NO <sub>2</sub> <sup>-</sup> - NaNO <sub>2</sub> . Arrows indicate changes in eubacterial genes for N+Ac; L and N + L treatments.(Courtesy of Prof. J. Prosser, Univ. of Aberdeen).....	
	122



- Figure 47 b. DGGE analysis of 16S rRNA genes amplified from extracted DNA using Nitrobacter and AOB primers. C – control, N –  $\text{NH}_4\text{NO}_3$ , L – lime, Act – acetate,  $T_0$  – initial value, Nit –  $\text{NaNO}_2$ . B1 to B6 correspond to 6 different Nitrobacter sp. (Courtesy of Prof. J Prosser, Univ. of Aberdeen) ..... 122
- Figure 48. Phylogenetic tree for Nitrobacter like species isolated from soil incubations, + and – denote positive and negative correlations with the following treatments C – Control; L – Lime; N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate. Additional treatments refer to enrichment cultures isolated from Sourhope soil. (Figure courtesy of Prof. J. Prosser, Univ. of Aberdeen)..... 124
- Figure 49. Changes in the DGGE banding profiles for  $\beta$  AOB, Nitrobacter sp. And Eubacterial primers relative to control soil banding profiles. . (C – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate) ..... 125
- Figure 50. Sourhope field soil temperature (b); moisture content (c) and rainfall (d) daily means logged using an AWS from April 2000 until November 2001. Soil measurements of WFPS (a) were made during inorganic N sampling (N =  $\text{NH}_4\text{NO}_3$ , L =  $\text{CaCO}_3$ , C = control, N+L =  $\text{NH}_4\text{NO}_3$  +  $\text{CaCO}_3$ ). ..... 139
- Figure 51. Effects of land management on nitric and nitrous oxide flux in the field. Arrows indicate treatment application dates, C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$ , N+L =  $\text{NH}_4\text{NO}_3$  +  $\text{CaCO}_3$  (Error bars =  $\pm$  S.E., n=3)..... 141
- Figure 52. The effects of land management treatment applications in the field on soil exchangeable ammonium (a) and nitrate concentrations (b). Arrows indicate treatment application dates (C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$ , N+L =  $\text{NH}_4\text{NO}_3$  +  $\text{CaCO}_3$ )..... 144
- Figure 53. Regression model of nitric (a) and nitrous oxide (b) emission from an upland agricultural grassland soil following N addition indicated by the arrows (C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$  and N+L =  $\text{NH}_4\text{NO}_3$  +  $\text{CaCO}_3$ ) ..... 147

Figure 54. Diurnal field measurements of nitrogen di-oxide (a) nitric oxide (b) and soil and air temperature (c) over a 24 hour period from the 29th to the 30th September 2001 from treatment block 3 at Sourhope (C = control, L = $\text{CaCO}_3$ , N = $\text{NH}_4\text{NO}_3$ , N+L = $\text{NH}_4\text{NO}_3 + \text{CaCO}_3$ ). .....	149
Figure 55. Relationship between soil WFPS and the ratio of NO emissions to $\text{N}_2\text{O}$ emissions for fluxes measured in chapter 4. The solid line is the regression ( $R^2 = 40.9\%$ , $P < 0.0001$ ). .....	158



## List of Tables

Table 1. Fractionation factors ( $\alpha$ ) for processes in the nitrogen cycle due to kinetic fractionations (Höberg, 1997).....	28
Table 2. Treatment application dates and mowing schedule at the Sourhope Rigg Foot field site .....	37
Table 3. Apparent $Q_{10}$ values for nitric and nitrous oxide emissions in soil microcosms measured on day one following addition of $\text{NH}_4\text{NO}_3$ at 40 and 80% WFPS .....	81
Table 4. Treatment applications made and processes inhibited by them.....	90
Table 5. Summary of treatment additions to soil microcosms, control treatments received de-ionised water only.....	104
Table 6. Effects of one week treatment additions, ANOVA Tukeys pairwise comparison tests were determined for 95% confidence intervals (Minitab, USA). Statistically significant treatment differences are denoted by a * for nitric oxide flux data and + for nitrous oxide flux data.....	112

## List of Equations

Equation 1. Atmospheric nitric oxide reactions and production of acid rain.....	8
Equation 2. Atmospheric nitric oxide reactions and production of acid rain.....	8
Equation 3. Calculation of soil WFPS .....	21
Equation 4. Isotopic fractionation factors for unidirectional reactions.....	27
Equation 5. Isotopic fractionation factors for equilibrium reactions .....	27
Equation 6. Nitric oxide reactions with ozone .....	38
Equation 7. Flux density calculations for nitric oxide emissions.....	45
Equation 8. Flux density calculations for nitrous oxide emissions.....	47
Equation 9. Regression equation for 0-100 sccm MFC .....	57
Equation 10. Regression equation for 0-2.5 slpm MFC .....	57
Equation 11. Gross nitrification rate calculations of Barraclough and Puri (1995)....	91
Equation 12. CO <sub>2</sub> requirement for autotrophic nitrification .....	107
Equation 13. Moles of CO <sub>2</sub> utilised for autotrophic ammonium oxidation .....	108
Equation 14. Moles of CO <sub>2</sub> utilised for autotrophic nitrite oxidation .....	108
Equation 15. Linear regression equation for fractionation factors of N <sub>2</sub> O formation.....	119
Equation 16. Stepwise linear regression equation for nitrous oxide emissions .....	145
Equation 17. Stepwise linear regression equation for nitric oxide emissions.....	145

## List of Plates

Plate 1. Sourhope Rigg Foot Soil Biodiversity site from Block 1A .....	32
Plate 2. Soil profile from Sourhope control plots 0-10 cm depth. ....	32
Plate 3. Field chamber and sampling for nitrous oxide using a 500 ml Hamilton syringe and 1 L Tedlar bag. ....	39
Plate 4. NO flux measurement system for soil microcosm experiments, mass flow controllers are indicated by black arrows with direction of air flow with red arrows. ....	45
Plate 5. Soil microcosm set-up for long term soil incubation and $^{13}\text{C}$ pulse labeling. .....	105

## Chapter One. Introduction

### 1.1.The global nitrogen cycle

The cycling of nitrogen has, in recent times, become of greater environmental significance due to the anthropogenic impacts if the nitrogen losses to the atmosphere. Mineral and organic nitrogen (e.g. Urea from cattle) are incorporated into both terrestrial and aquatic environments and form the nitrogen (N) cycle (Figure 1.). The largest reservoir for nitrogen is the atmosphere where it constitutes 79% of the gaseous atmosphere as di-nitrogen gas ( $N_2$ ). The microbial biogeochemical cycling of elemental nitrogen is of fundamental importance to a sustainable ecosystem, microorganisms play an important role in many of the processes shown in Figure 1. and provide one of the few natural mechanisms by which nitrogen can enter the ecosystem.

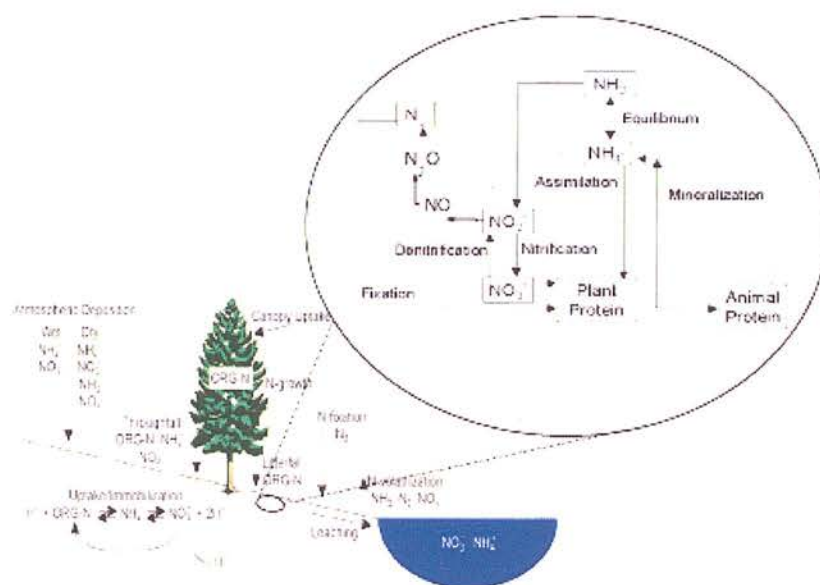


Figure 1. Nitrogen cycle



### 1.1.1. Di-Nitrogen fixation

All habitats depend upon the fixation of atmospheric nitrogen by bacteria for their cellular nitrogen, although other sources of N include industrial fixation and fertilisation as well as fixation by fungi (Atlas and Bartha, 1998). The bacteria responsible for this process of N fixation include free living species found mainly in the soil rhizosphere (e.g., *Azotobacter*, *Clostridium*, *Methanococcus* and *Pseudomonas*) as well as symbiotic microbial associations with plants (*Rhizobium*, actinomycetes and cyanobacteria).

The symbiotic fixation of nitrogen involves the invasion (infection) of plant root hairs by nitrogen fixing bacteria such as *Rhizobium spp.*, this is followed by formation of a tumour-like nodule within which the bacteria produce the nitrogenase enzyme and fix atmospheric nitrogen.

There are several genera of free living species of bacteria and fungi that are capable of fixing atmospheric nitrogen, including members of the actinomycetes. The rates of nitrogen fixation by free living species of bacteria are relatively low compared with the rates of nitrogen fixation by symbiotic relationships, typically one third of the rates for symbiotic nitrogen fixing species (Atlas and Bartha, 1998). In both systems the enzyme responsible for fixation of molecular nitrogen is the nitrogenase enzyme, the regulation for production of this enzyme is complicated and is governed by both extracellular plant exudates and microbial cell surface receptors (Atlas and Bartha, 1998). The symbiotic species of nitrogen fixing bacteria require anaerobic conditions for nitrogenase enzyme functioning, which is provided by the plant (Prescott *et al.*, 1993). The nitrogen that is fixed is incorporated into the plant biomass as organic nitrogen via the glutamine synthetase – glutamate synthase system.

### 1.1.2. Organic nitrogen mineralisation

Dead plant litter, animal excreta and dead microbial biomass are all relatively immobile in that they have high C:N ratio's and therefore represent more complex forms of carbon for example lignin, which require mineralisation in order to release

more available forms of nitrogen in the soil. Mineralisation is the release of mineral N from organic compounds as ammonium. This process is almost entirely due to microbial activity, and in particular heterotrophic ammonification. Soils with high C:N ratios (25-30:1) are generally slow to release inorganic nitrogen and hence may result in immobilisation of nitrogen, where inorganic nitrogen is converted to an organic form in microbial biomass making the nitrogen unavailable to other organisms. Immobilisation has the potential to reduce nitrification rates in soil by removing ammonium as a substrate (Whitehead, 1995).

Earthworms and other soil fauna promote mineralisation in soils by consumption of organic matter in leaf litter and subsequent incorporation into their biomass. The effects of earthworms on mineralisation rates become less significant as a result of inorganic N addition to soils (Bohlen *et al.*, 1999). The process of mineralisation tends to result in N loss from soil via nitrification and denitrification especially in soils with relatively low C:N ratios which reflects the already high soil N content and possibly more dominant autotrophic nitrifying species of bacteria relative to other soil bacteria such as the organic carbon requiring heterotrophs (Whitehead, 1995).

Heterotrophs require organic forms of carbon to obtain energy and for biomass whereas autotrophs use inorganic forms of nitrogen to obtain energy and carbon dioxide rather than organic carbon is incorporated into the biomass, a lower C:N ratio therefore results in less carbon available for heterotrophic growth and therefore autotrophs are able to out compete the heterotrophs for terminal electron acceptors. Fertiliser addition to grazed grassland systems can result in significant increases in net N mineralisation, where mineralisation and production of ammonium exceeds the rate at which it is nitrified. However maximum mineralisation rates can often occur several months after additions are made which is known as the priming effect, this is the result of increased concentration of extracellular enzymes which are produced by microorganisms resulting in accelerated decomposition of organic forms of nitrogen (Lavelle and Gilot, 1994), this may also coincide with slow microbial generation times and increased numbers of nitrifying bacteria as a result of increased substrate availability (Mills and Watson, 1997).

The process of immobilisation is the reverse of mineralisation and involves the assimilation of organic nitrogen into the microbial biomass which is carried out by assimilatory nitrate reducing organisms.

### 1.1.3. Nitrification

Nitrification is the oxidation of ammonium to firstly nitrite and then nitrate. These are two distinct processes of the nitrification pathway carried out by two distinct groups of microorganisms, ammonium oxidation is carried out by *Nitrosomonas* sp. and nitrite oxidation to nitrate by *Nitrobacter* sp.. Nitrification is a predominantly aerobic process and becomes inhibited at low partial pressures of oxygen, which can be brought about by high soil moisture contents (Robertson and Tiedje, 1987). The process of nitrification occurs in the majority of soil types but is a major process involved in the production of nitrous oxide in agricultural soils (Conrad, 1995).

Heterotrophic nitrifiers utilise organic and inorganic nitrogen in the form of ammonium and obtain their cellular carbon from organic carbon, autotrophic nitrifiers on the other hand utilise inorganic nitrogen and obtain their cellular carbon from CO<sub>2</sub> (Conrad, 1995).

### 1.1.4. Denitrification

Denitrification requires mainly anaerobic conditions for the complete reduction of nitrate to gaseous nitrogen (N<sub>2</sub>) via nitrite and nitric oxide and nitrous oxide. In soil two dominant genera capable of denitrification are *Pseudomonas* and *Alcaligenes*, although other genera such as *Azospirillum*, *Rhizobium*, and *Rhodopseudomonas* are also able to denitrify (Atlas and Bartha, 1998). Denitrifiers obtain their source of carbon from the oxidation of organic matter and their energy from the reduction of nitrate by dissimilatory nitrate reductases.

Assimilatory nitrate reduction (ANR) involves the incorporation of nitrate ions into cellular organic matter; this is carried out by a heterogeneous group of



organisms including bacterial and fungal species, and involves the nitrate and nitrite reductases, to form ammonium which is then incorporated into amino acids (Atlas and Bartha, 1998). The enzyme systems of ANR are repressed by high concentrations of ammonium in the growth environment and are unaffected by atmospheric oxygen concentrations (Zumft, 1997).

Denitrification is an important source of  $N_2O$  in wetland soils and is often coupled to nitrification in upland soils where aerobic and anaerobic microsites allow simultaneous production and consumption of nitrate by nitrifiers and denitrifiers, respectively.

### 1.1.5. Leaching

The leaching of inorganic nitrogen occurs when soils at field capacity receive further rainfall that then moves downwards through the soil profile. Nitrate is an inorganic form of nitrogen which undergoes leaching from terrestrial ecosystems, most frequently during the winter. The nitrate in soils is particularly susceptible to leaching because it is not absorbed by clay and organic colloids which are negatively charged, and represents a loss of plant available N and also nitrate available for denitrification. Increased use of nitrogen based fertilisers has resulted in an increase in nitrate leaching and with it increases of nitrate in groundwaters. This has major implications for aquatic ecosystems in terms of eutrophication. Leaching is affected by the soil structure and porosity, with large pores ( $>0.05$  mm) allowing increased rates of water movement through the soil (Whitehead, 1995).

## 1.2. NO and $N_2O$ atmospheric budget (sources sinks and sizes, acid rain)

Nitric and nitrous oxide are both important products of nitrification and denitrification, their atmospheric sources and sinks have still to be fully understood with conflicting sizes of sources and sinks, which thus far have not been accounted for (Nevison *et al.*, 1999).

### 1.2.1. Nitric oxide

Nitric oxide is an extremely reactive gas in the atmosphere and a major source of atmospheric ozone (Matson, 1997; IPCC, 2001). Soils represent a relatively large non-anthropogenic source of  $\text{NO}_x$  ( $\text{NO} + \text{NO}_2$ ) and  $\text{NO}_y$  ( $\text{NO} + \text{organic nitrates} + \text{inorganic nitrates}$ ) (Davidson and Kingerlee, 1997; Fehsenfeld D., 1995). Sources for nitric oxide are varied and include anthropogenic driven production via biomass burning ( $2\text{--}40 \text{ Tg N yr}^{-1}$ ) and fossil fuel combustion ( $8\text{--}28 \text{ Tg N yr}^{-1}$ ) (Conrad, 1990). Soils are a significant natural source of nitric oxide ( $1.1\text{--}21 \text{ Tg N yr}^{-1}$ ) (Davidson and Kingerlee, 1997), with greater production from acidic (pH 4.7) soils as opposed to less acidic soils (pH 5-6) where gross production rates were  $7.6$  and  $1.4 \text{ ng N h}^{-1} \text{ g soil}^{-1}$ , respectively (Remde *et al.*, 1989). With the increased use of nitrogen based fertilisers agricultural grasslands have become an important source of NO with flux in acidic soils (pH 3.9) showing greater emission rates ( $1.15 \text{ ng N m}^{-2} \text{ s}^{-1}$ ) (Yamulki *et al.*, 1997) making land management issues an important part of governmental environmental policy. Present atmospheric mixing ratios are less than 1 ppbv (parts per billion per volume), where the mixing ratio corresponds to the ratio of the volume of the gas of interest to the volume of the air which it is in (1 ppm ( $10^6$ ) is  $1\text{cm}^3$  of the gas per  $10^6 \text{ cm}^3$  of air). The units of ppb ( $10^9$ ) assume ideal gas behavior and therefore concentration is not affected by temperature and pressure as they affect both the gas of interest and the air which it is in to the same extent.

However; due to the extremely reactive nature of nitric oxide with other atmospheric components such as ozone relatively large fluxes of nitric oxide are needed to maintain these low mixing ratios (Conrad, 1990).

The oxidation of ammonia by nitrifying bacteria and reduction of nitrate by denitrifying bacteria seem to be the most important processes involved in NO production in soils (Remde & Conrad, 1991). Skiba *et al.* (1992) found that nitrification was the dominant process in NO emission from a sandy loam soil which showed a significant correlation with several environmental variables.

Although soils are usually sources of NO in the atmosphere they also occasionally act as sinks for NO (Krämer and Conrad, 1991) where NO is both

produced and consumed by nitrifying and denitrifying bacterial populations (Remde *et al.*, 1989; Remde & Conrad, 1991; Rudolph *et al.*, 1996).

### 1.2.2. Nitrous oxide

Nitrous oxide concentrations have increased in the atmosphere from 275 ppbv during the pre-industrial era to 317 ppbv in 1992 (IPCC, 2001) and soils have been found to account for more than 60% of the known global sources of nitrous oxide (Figure 2.)

There are however large uncertainties in the sources and sink of nitrous oxide with current total source estimates between 6.7 and 36.6 Tg N yr<sup>-1</sup> (IPCC, 2001) and total sink estimate between 9 and 16 Tg N yr<sup>-1</sup>, these uncertainties are in part due to large spatial and temporal variations in emissions. Tropical soils which tend to be phosphorous limited result in much larger emissions of nitrous oxide (10 to 100 hundred fold greater) when fertiliser N is applied than in an N limited terrestrial system (Hall and Matson, 1999), these larger fluxes for tropical soils add to the uncertainties for sources of nitrous oxide (IPCC, 2001)



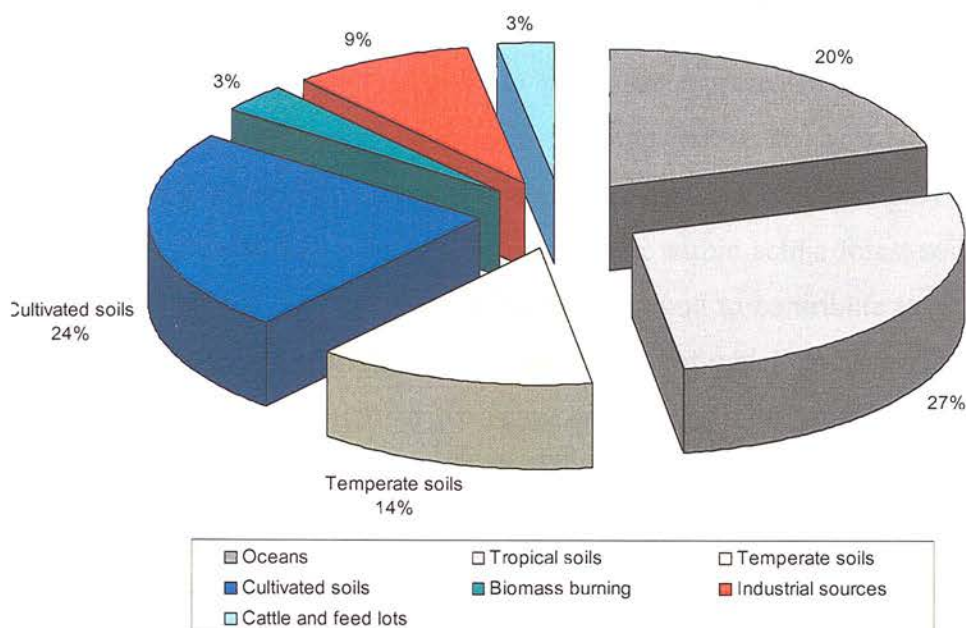


Figure 2. Global sources of  $N_2O$  ( $Tg\ N\ yr^{-1}$ ) (IPCC, 1994)

As well as being a radiatively forcing greenhouse gas, nitric oxide has been implicated as a cause of acid rain which occurs via a series of reactions shown in Equations 1 and 2.

Equation 1. Atmospheric nitric oxide reactions and production of acid rain



Equation 2. Atmospheric nitric oxide reactions and production of acid rain



The atmospheric lifetime of  $\text{N}_2\text{O}$  has been estimated at around 120 years (Graedel and Crutzen, 1989) and present increases have been attributed to an increase in the use of N fertilisers in agriculture (Matson and Vitousek, 1990).

Microbial processes in soils in particular represent a major source of  $\text{N}_2\text{O}$  (Banin, 1986), production of which is important from both nitrification and denitrification especially in upland soils (Conrad, 1995). Not only bacteria are responsible for nitrous oxide production in soils, within acidic forest soils organisms other than nitrifiers and denitrifiers have been found to contribute to the production of  $\text{N}_2\text{O}$  (Robertson and Tiedje, 1987), production of which could be linked to lignin degradation and production of oxidising radicals by nitrifying heterotrophic fungi (De Boer and Kowalchuk, 2001). Grasslands could possibly be one of the most significant sources for potential increases in the nitrous oxide production from soils, as a result of land management practices and decreasing acidity with liming, Yamulki *et al.* (1997) found that within acidic grassland soils  $\text{N}_2\text{O}$  production decreased with increasing acidity.

### 1.3. $\text{NO}_2^-$ , $\text{NO}_3^-$ and $\text{NH}_4^+$ terrestrial budget

The concentrations of nitrite, nitrate and ammonium and their subsequent global budgets have not been extensively reviewed due to the fact that they are all very transient dynamic nutrients, and are important as intermediates of nitrogen cycling processes. These soluble inorganic forms of nitrogen are relatively short lived in the terrestrial environment and are either utilised by microbial populations, undergo chemical oxidation or are leached out into streams and rivers where they are ultimately involved in the eutrophication of lakes and reservoirs (Whitehead, 1995).

### 1.4. Importance of biodiversity on the soil nitrogen cycle

The concept of biological diversity has become extremely popular within mainstream science and even the tabloid press, although not always accurately reported in the latter. Biodiversity is a concept which has been devised to try and

quantify the variety of life, the word itself is a contraction of “biological” and “diversity” meaning “the study of life” and “variety” respectively. Biodiversity is becoming increasingly important in a world which is being shaped by the effects of man.

Research has been directed at quantifying the biodiversity of microbial populations in terrestrial ecosystems (Brussard *et al.* 1996; Pankhurst *et al.*, 1996; Ward *et al.*, 1990). Little has been done to try and link this microbial biodiversity with microbial functioning (Torsvik, 1998). Previous measures of biodiversity have been based on culturable bacteria; however only 1 to 5% of the whole microbial population has been classified and grown on standard media (Ward *et al.*, 1990; Kennedy & Gewin, 1997), the true biodiversity *in situ* will never be quantified using culturable techniques.

The importance of a diverse microbial community is evident in that, with low species dominance there will be a greater genetic diversity and hence rapid and varied responses to any perturbations of the environment, maintaining ecosystem functioning (Tillman & Downing, 1994). Several studies have focussed on higher trophic levels, looking at the plant species diversity in grassland ecosystems one of which is the Park grass experiment (Grime *et al.*, 1987).

Biodiversity is deemed to be the most important ecosystem property when it is subjected to an environmental perturbation (McGrady-Steed *et al.*, 1997). It is this biodiversity that will determine the way in which the ecosystem will function in response to a perturbation (Grime *et al.*, 1987; Tillman and Downing, 1994; Tillman *et al.*, 1996; McGrady-Steed *et al.*, 1997). However, high species diversity does not always reflect the measure of ecosystem functioning and vice versa, in ecosystems where physicochemical factors are the major controlling factors such as acid bogs and hot springs, the evolution and balance of closely related species interactions are not as important (Atlas and Bartha, 1998).

The use of 16SrRNA gene sequences provides the basis for phylogenetic classification of microorganisms (Prosser and Embley 2002), 16SrRNA sequence analyses of ammonia oxidisers has identified both  $\gamma$  and  $\beta$  – proteobacteria from



environmental samples (Prosser and Embley, 2002); with *Nitrosomonas* clusters 5-7 and *Nitrospira* clusters 1-4 falling within the  $\beta$  – proteobacteria and *Nitrosococcus* within the  $\gamma$  – proteobacteria (Teske, 1993).

The molecular diversity of organisms within the nitrogen cycle has been assessed using key enzymes in the pathways of nitrification and denitrification to target specific functional genes (Mendum *et al.*, 1999). Specific PCR primers encoding the ammonium monooxygenase enzyme and in particular the *amoA* gene has enabled further identification of putative ammonia oxidisers from environmental samples to be possible (Purkhold *et al.*, 2000).

The functional diversity of soil microorganisms has been quantified using substrate induced respiration, Biolog plates and extracellular enzyme assays (Degens *et al.*, 2001; Garland and Mills, 1994; Nannipieri *et al.*, 2003).

In studies measuring both function and diversity, the links between the two may not always be clear cut; Mendum *et al.* (1999) looked at the effects of nitrogen fertiliser additions on autotrophic ammonia oxidiser populations in an arable soil, they noted that rates of nitrification increased significantly following fertiliser additions but that size of the ammonia oxidising population remained unchanged until 6 weeks later when process rates had decreased again. Recent studies have used both process rates and  $^{13}\text{C}$  DNA extraction techniques to link diversity directly with function (Radajewski *et al.*, 2000),  $^{13}\text{C}$  labelled  $\text{CO}_2$  was used by Whitby *et al.* (2001) to label 16S rDNA of autotrophic nitrifiers and thus identify active  $\text{CO}_2$  respiring ammonia oxidising bacteria in fresh water sediments, they found that  $^{13}\text{C}$  incorporation into nitrosomonad DNA was greater than that of nitrospira DNA indicating that nitrosomonads are able to out compete the nitrospiras for carbon linking diversity to function.

The emissions of nitric and nitrous oxide are linked to process rates of both nitrification and denitrification (Firestone and Davidson, 1989), few studies have looked at nitric and nitrous oxide emissions and the links with changes in molecular diversity.

### **1.4.1. Quantification of microbial biodiversity**

Microbial diversity can be measured at several different levels that relate to function and physiology. Diversity at the species level i.e. species of bacteria present will provide information at the ecosystem scale, whereas genetic diversity can provide information on the community and individual scale in terms of functioning. It is difficult to determine the true microbial diversity and at what rate the true species diversity will change in response to a treatment addition.

The measure of species diversity has generally been in the form of species diversity indices, where the numbers of species and their relative abundance's have been quantified and given a single number to quantify the diversity. There are several diversity indices which take into account two factors: species richness (number of species) and evenness (how equally abundant are the species). The difference between the respective indices is in the weighting they give to evenness and species richness (Magurran, 1988). The use of such indices may not be appropriate for the scale (molecular) on which microbial diversity indices should be based. Molecular diversity is often representative at the functional gene scale, in which case many species of bacteria could potentially be classified as one in terms of their functional molecular diversity should they possess the gene but not express it in terms of actual function.

## **1.5. Importance of nitrogen cycling to global climate change**

### **1.5.1. IPCC / Kyoto protocol / current state of affairs**

The Inter-governmental Panel on Climate Change (IPCC) was established in 1988 jointly by the World Meteorological Organisation and the United Nations Environment programme, with a remit that was to ultimately lead to an internationally binding convention for protecting the environment. The Parties to the Convention have agreed by consensus (Dec. 1997) that developed countries will have a legally binding commitment to reduce their collective emissions of six

greenhouse gases by at least 5% compared to 1990 levels by the period 2008-2012 (UNFCCC, 1999). The Kyoto Protocol also allows for an emissions trading regime and a "clean development mechanism", allowing the reduction in emissions of several greenhouse gases to be credited against a CO<sub>2</sub>-equivalent emissions limit, these are calculated using global warming potential indices for each gas (Reilly *et al.*, 1999). A copy of the Kyoto Protocol can be found at United Nations Framework Convention on Climate Change (IPCC, 2000).

Climate change can be defined as changes in temperature, rainfall and associated meteorological variables (Cannell *et al.*, 1999) which may or may not be the result of human activities. Carbon dioxide concentrations in the atmosphere are expected to reach 500 ppm by the end of the 21<sup>st</sup> century using present climate change models. To stabilise nitrous oxide concentrations at today's levels would involve reductions in anthropogenic emissions of more than 50% (IPCC, 1998).

### 1.5.2. Radiative forcing by oxides of nitrogen

Radiative forcing is the change in average net radiation at the top of the troposphere due to a change in either solar or infrared radiation, this radiative forcing perturbs the balance between incoming and outgoing radiation. Nitrous oxide has a direct positive radiative forcing of the global climate whereas nitric oxides have an indirect radiative forcing on the global climate by influencing atmospheric ozone concentrations. The radiative forcing due to N<sub>2</sub>O has increased since the pre-industrial era to about 0.1 Wm<sup>-2</sup> (IPCC, 1994).

Nitrous oxide concentrations have been increasing over the past four with a growth rate of 0.25% yr<sup>-1</sup> decades (IPCC, 1994). This increase is mainly due to the expansion and intensification of agricultural activities i.e. increased use of N fertilisers (Smith, 1997).

Nitric oxide and nitrogen dioxide are both extremely reactive in the atmosphere and take part in reactions with atmospheric ozone where nitric oxide is oxidised to NO<sub>2</sub> by ozone, during the daytime this NO<sub>2</sub> is photolysed at wavelengths



of light  $<420$  nm back to NO (Figure 3). Further reactions involving nitrogen dioxide are shown in Figure 4., where NO<sub>2</sub> reacts with free hydroxyl radicals in the atmosphere to form HNO<sub>3</sub> which is rapidly incorporated into cloud water, this is then deposited to the ground and is a major component of acid rain (Conrad, 1990). The atmospheric lifetime of NO<sub>x</sub> is relatively short lived 1-10 days whereas the lifetime of N<sub>2</sub>O is much longer ( $\sim 120$  years) (Olivier *et al.*, 1998; IPCC 2001).



Figure 3. Atmospheric chemistry of nitric oxide and nitrogen dioxide with ozone.



Figure 4. Production of nitric acid via complex interconversion sequences.

Nitrous oxide has a direct effect on the radiative forcing in the atmosphere and is also a source of stratospheric NO<sub>x</sub> as shown in Figure 5. (IPCC, 2001).

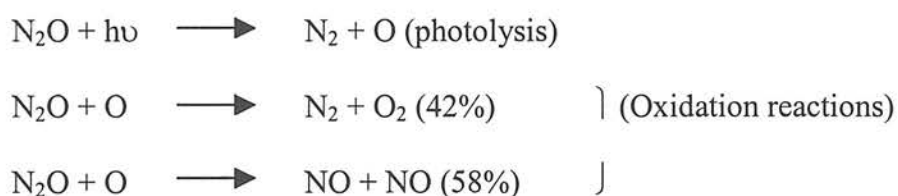


Figure 5. Stratospheric sinks for nitrous oxide

The oxidation reactions of N<sub>2</sub>O in the stratosphere shown in Figure 5 are divided into three chemical reactions, with the total sink for N<sub>2</sub>O being the sum of the three (Nevison *et al.*, 1999). However, it is possible that the current

understanding of the global budget for  $\text{N}_2\text{O}$  is flawed in that further reactions with ozone have not been taken into account, these reactions may effectively increase the indirect atmospheric lifetime of  $\text{N}_2\text{O}$  as a radiatively active gas (Prather, 1998).

## 1.6. Production and consumption of NO and $\text{N}_2\text{O}$ in soils

Soil microbial processes are generally regarded as the major global sources of  $\text{N}_2\text{O}$  and  $\text{NO}_x$  (Banin, 1986; Baumgärtner and Conrad, 1992). Firestone and Davidson (1989) proposed a model (Figure 6.) indicating two levels of regulation for the production of NO and  $\text{N}_2\text{O}$  via nitrification and denitrification. They suggested that the two processes work like a series of pipes through which nitrogen transformation occurs, the holes in the pipes are through which NO and  $\text{N}_2\text{O}$  leak.

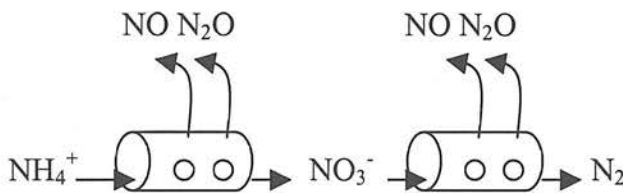


Figure 6. Model of nitrogen flux as proposed by Firestone and Davidson (1989).

### 1.6.1. Nitrification (autotrophic/heterotrophic)

The process of nitrification in soils involves the oxidation of a relatively immobile ( $\text{NH}_4^+$  tightly bound to clay particles via cation exchange) to an extremely mobile ( $\text{NO}_3^-$ ) stage via nitrite, this provides opportunities for “leakage” of other nitrogenous compounds including  $\text{N}_2\text{O}$  and NO (Jarvis, 1996/1997). The initial step of nitrification (Figure 7.) involves the oxidation of ammonia to hydroxylamine, followed by further oxidation reactions involving hydroxylamine oxidoreductase to form nitrite via an intermediate bound nitric acid and hydrogen from hydroxylamine and water. The production of nitric and nitrous oxide via the oxidation of hydroxylamine may be due to slow electron transfer by this enzyme and a need to

prevent nitrite accumulation to toxic concentrations within the cell or production of another as yet unknown compound (X) which could then be oxidised to nitrite and nitric oxide (Ferguson, 1998; Khalil *et al.*, 2004). The production of hydrogen ions reduces the pH of the environment in which nitrification occurs and also provides a reduced soil redox potential (Anderson *et al.*, 1993 and Rudolf *et al.*). Although the second step of nitrification is oxygen dependent it obtains its oxygen for the formation of nitrite from a water molecule and not molecular oxygen (Nevison *et al.*, 1999). The soil nitrifying microorganisms not only produce NO directly, but also increase the relative importance of NO production from  $\text{NO}_2^-$  and  $\text{NO}_3^-$  via soil chemical reactions (chemodenitrification) and also denitrification and nitrifier denitrification of nitrite (Hutchinson *et al.*, 1997, Wrage *et al.*, 2003). Heterotrophic nitrification (Figure 8.) involves the oxidation of organic nitrogen as a terminal electron acceptor.

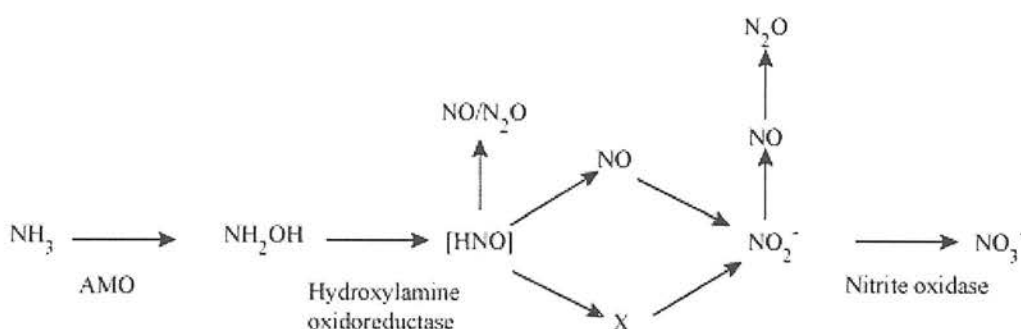


Figure 7. The autotrophic nitrification pathway



Figure 8. The heterotrophic nitrification pathway

Nitric and nitrous oxide can be produced from both autotrophic and heterotrophic nitrification, Anderson *et al.* (1993) reported that autotrophic nitrifiers such as *Nitrosomonas europaea* are the predominant producers of NO and  $\text{N}_2\text{O}$  in aerobic soils whereas heterotrophic nitrifiers such as *Alcaligenes faecalis* are more important producers of these gases in near anaerobic soils. Others have linked the

soil pH and hence redox potential of soils with the dominant source of NO and N<sub>2</sub>O (Anderson *et al.*, 1993; Yamulki *et al.*, 1997). The flux of NO from various soil types after fertiliser treatment addition ranged from 1.19 ng N m<sup>-2</sup> s<sup>-1</sup> in a clay loam soil to 4.21 ng N m<sup>-2</sup> s<sup>-1</sup> in a sandy loam (McKenney & Drury, 1997), this indicates that soil type is an important factor affecting rates of nitrogen loss from soils.

The production of NO seems to be predominantly due to, or dependant upon rates of nitrification in soils (Vermoesen *et al.*, 1996), and hence the rate at which NO leaks out of the pipes (Firestone and Davidson, 1989).

The production of NO via *N. europaea* may be due to a putative denitrification pathway in which nitrite is used as an electron acceptor and leads to production of NO and N<sub>2</sub>O, at low oxygen concentrations nitrite oxidoreductase is inhibited and this dissimilatory reduction of nitrite may be a mechanism to prevent nitrite accumulating within the cell (Beaumont *et al.*, 2002), under anaerobic conditions nitrite has also been shown to act as an electron acceptor with hydroxylamine oxidation, while nitrous oxide was produced (Bruijn *et al.*, 1995).

Heterotrophic nitrification is particularly important in production of N<sub>2</sub>O in acidic soils which was found to be due to organisms other than nitrifiers and denitrifiers and could be attributed to nitrifying acidophilic fungi (Robertson and Tiedje, 1987). This however is not the case for all soil types (Inubushi *et al.*, 1996) and in upland agricultural soils nitrification is a predominant source of N<sub>2</sub>O (Robertson and Tiedje, 1987 and Conrad, 1995).

### 1.6.2. Denitrification (autotrophic/heterotrophic)

The denitrification pathway has both NO and N<sub>2</sub>O as intermediates and hence the potential to be a major source of atmospheric nitrogen and in particular N<sub>2</sub>O under decreasing soil pH (Nägele and Conrad, 1990). Denitrifying organisms have a broad range of activity and are active from pH 5 to 8, in soils with high nitrate concentration pH has a greater influence on the gaseous product ratio of N<sub>2</sub>O:N<sub>2</sub> (Yamulki *et al.*, 1997). Denitrification is carried out by heterotrophic and autotrophic groups of microorganisms, heterotrophic denitrification involves the assimilation of



organic carbon into the cell biomass with nitrate used as an energy source (Figure 9.).

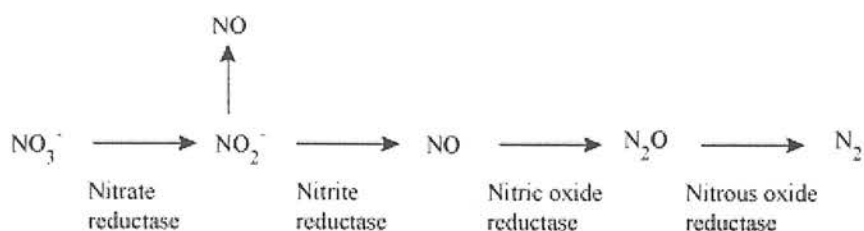


Figure 9. The denitrification pathway

The production of NO via denitrification does not represent a major emission product, probably due to a much reduced environment where complete reduction of nitrate to  $\text{N}_2\text{O}$  or  $\text{N}_2$  is possible (Anderson and Levine, 1986).

Soil moisture content, organic carbon and nitrate availability all significantly affect the gaseous products of denitrification (Conrad 1996; Swerts *et al.*, 1996). The enzymes in the denitrification pathway are affected by oxygen concentrations mediated by soil moisture content, each of the enzymes are expressed sequentially with decreasing oxygen; nitrate reductase is the least sensitive to oxygen followed by NO and  $\text{N}_2\text{O}$  reductases and finally nitrite reductase; with nitrite reductase being the most sensitive to oxygen (Zumft 1997; Ferguson 1994). The product ratio of both  $\text{NO}:\text{N}_2\text{O}$  and  $\text{N}_2\text{O}:\text{N}_2$  are therefore influenced by soil moisture to varying degrees, under slightly aerobic conditions denitrifiers may be able to reduce nitrite produced via nitrification to NO thus increasing the  $\text{NO}:\text{N}_2\text{O}$  via a form of coupled nitrification denitrification with increasing soil moisture content the  $\text{NO}:\text{N}_2\text{O}$  ratio will decrease and the  $\text{N}_2\text{O}:\text{N}_2$  ratio will increase as the denitrification enzymes synthesised via transcription and translation increase.

Nitrate has been shown to result in increased denitrification in soils (Šimek *et al.*, 1999), and indeed nitrate is required for induction of denitrification (Zumft, 1997). The nitrate reductase and nitrite and nitrous oxide reductase genes may also be activated by nitrate, with nitric oxide reductase however expression is concomitant with other denitrification enzymes and therefore would result in lower NO:N<sub>2</sub>O and accumulation of N<sub>2</sub>O this increasing the N<sub>2</sub>O:N<sub>2</sub> ratio. Nitrous oxide reduction to N<sub>2</sub> would require completely anaerobic conditions and therefore with addition of nitrate the N<sub>2</sub>O:N<sub>2</sub> ratio increases and the NO:N<sub>2</sub>O ratio decreases (Zumft, 1997)

Denitrifiers are heterotrophic organisms and thus require organic carbon as an electron donor (Zumft, 1997). When soil nitrate concentrations are low but organic carbon concentrations are high N<sub>2</sub> is the dominant end product of denitrification, the opposite is true when organic carbon is low when N<sub>2</sub>O is the dominant end product (Firestone, 1980). Thus organic carbon can affect the product ratio of denitrification, however it is probably that organic carbon is an indicator of more favourable conditions for denitrification

### 1.6.3. Dissimilatory/assimilatory nitrate reduction

N<sub>2</sub>O can be reduced by anaerobic bacteria that exhibit a dissimilatory reduction of nitrate to ammonium (DNRA) such as *Wollinella succinogenes* (Teraguchi and Hollocher, 1989; Conrad, 1995). The process of dissimilatory nitrate reduction occurs under anaerobic conditions where nitrate is used as an electron sink and the responsible bacteria are fermentative. DNRA occurs under similar conditions to denitrification, with high soil moisture content and highly reduced conditions. The partitioning between nitrate reduction to ammonium and denitrification is strongly influence by soil labile carbon (Yin *et al.*, 2002).

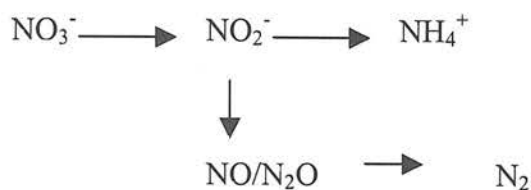


Figure 10. DNRA reaction sequence

#### 1.6.4. Chemodenitrification

Chemodenitrification is the abiological formation of oxides of nitrogen, and generally is only a minor source for  $\text{N}_2\text{O}$  (Conrad, 1996) in which hydroxylamine is decomposed. The chemical production of NO on the other hand can be much greater. Production of NO via non-biological processes tends to be due to increased soil nitrite concentrations where nitrite is protonated to form nitrous acid ( $\text{HNO}_2$ ) at low soil pH which can subsequently decompose in aqueous solution to form NO, chemodenitrification becomes more important in acidic soils where nitrite and hence nitrous acid may accumulate NO (Venterea and Rolston, 2000).

Nitrite concentrations in soils are usually low and rarely exceed  $50 \text{ mg NO}_2^- - \text{N kg}^{-1}$  soil; it is possible for nitrite in soils to be decomposed to gaseous forms of nitrogen under acidic conditions by spontaneous decomposition of nitrous acid (Van Cleemput & Samater, 1996).

### 1.7. Factors affecting NO and $\text{N}_2\text{O}$ emission and production in soils

#### 1.7.1. Soil water content (WFPS)

The soil water content is an important environmental variable that has major implications for production and emission of both  $\text{NO}_x$  and  $\text{N}_2\text{O}$  via nitrification and denitrification. Soil moisture has typically been expressed as water potential (Stark and Firestone, 1995); however this gives very little information about soil structure

in determining the moisture content and makes comparisons between different soil types difficult. Soil can be visualized as a three dimensional matrix with soil aggregates and a series of pore spaces in which water can accumulate. Soil water content is measured as the water filled pore space, gravimetric or volumetric soil water content and is dependent upon soil type and structure. Soil water content expressed as WFPS is determined using Equation 3, where the total soil porosity of a soil is the volume ratio of soil bulk density to particle density.

Equation 3. Calculation of soil WFPS

$$\text{WFPS} = \frac{\text{Volumetric soil moisture content}}{\text{Total soil porosity expressed as } 1 - (\text{bulk density/particle density})}$$

In aerobic soils nitrite is readily oxidised to nitrate via nitrification, however with a depletion of oxygen, nitrate is rapidly reduced to nitrite via denitrification to produce both nitric and nitrous oxide (Rosswall, 1982 and McKenny *et al.*, 1982). The sensitivity of soils to oxygen concentration is greater in agricultural soils than forest soils due to the effects of ploughing on soil structure and increased acidity in forest soils (Krämer & Conrad, 1991), with greater NO production from forest soils.

Not only does soil water content affect the microbial processes in the production and consumption of nitric and nitrous oxide it also affects gas transport into and out of the soil profile (Conrad, 1995), with diffusion of gases through water being much slower than through air as a result of solubility of gases and the Bunsen coefficient (Morris, 1974). Soil moisture content not only has a direct effect on microbial activity it also provides either anaerobic or aerobic microsites, bacterial synthesis of reduction enzymes in denitrifiers which are responsible for the sequential reduction of nitrate to ultimately N<sub>2</sub> is repressed under aerobic conditions and are oxygen sensitive (Dendooven and Anderson, 1995), oxygen affects the expression of promoter sequences for several denitrification enzyme genes to varying



degrees which to some degree explains the changing  $\text{N}_2\text{O}:\text{N}_2$  ratio as a function of anaerobicity (Zumft, 1997; Davidson, 1991). Denitrification may however also be carried out by aerobic denitrifiers, *Paracoccus denitrificans* being one example. The enzymes of aerobic and anaerobic denitrifiers are fundamentally identical, it is the non inhibition of enzyme regulation at the genetic levels that results in transcription of the reductase genes (Zumft, 1997). Summer rain events are of particular importance in that they mobilise a previously inactive group of bacteria with the potential for very high fluxes of NO and  $\text{N}_2\text{O}$  as a result of large soil nitrate pools produced as a result of aerobic nitrification.

### 1.7.2. Nitrogen availability (type and source)

The chemical properties of soils can be altered as a result of all three-treatment additions being made to the Sourhope field site, Šimek *et al.* (1999) showed that soil C and N contents increased with addition of inorganic fertiliser N although the C:N ratio was smaller in fertilised than in control plots, the organic carbon content increasing as a result of increasing biomass C.

The addition of fertiliser N alone on the other hand resulted in a decrease in the soil pH associated with increases in nitrifying bacteria and production of nitrite. In acid forest systems the rate of nitrification has been found to influence soil acidification mainly by heterotrophic nitrification (Haynes, 1986). The effects of nitrogen addition in grasslands results in losses of both NO and  $\text{N}_2\text{O}$  which normally account for less than 2% of the total N applied as fertiliser (inorganic), this is usually accompanied by a low pH (Vermoesen *et al.*, 1996).

The amount of inorganic nitrogen fertiliser determines whether or not there is net  $\text{N}_2\text{O}$  production or consumption. Ryden (1981) noted that application rates of  $250 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  resulted in consumption of  $\text{N}_2\text{O}$  under dry soil conditions with low nitrate concentrations, maximum sink activity was as high as  $11.6 \text{ ng N m}^{-2} \text{ s}^{-1}$ . Respiration and or reduction of nitrous oxide may be due to the low nitrate concentrations; under these conditions denitrifiers are able to use nitrous oxide as a

terminal electron acceptor for oxidation of organic compounds, other non denitrifiers are also able to respire nitrous oxide (Zumft, 1997).

Vermoesen *et al.* (1996) in similar experiments looked at the effects of fertiliser N additions (0, 50, 100 and 150 mg N kg<sup>-1</sup> soil) and found that fertiliser addition had a significant effect on nitric oxide production, and that the rate of nitrous oxide production was totally dependant on soil characteristics.

### 1.7.3. Soil pH (liming)

Liming has been shown to increase rates of nitrogen mineralisation in grassland soils (Whitehead, 1995), in studies of 40 soils ranging in pH from 4.0 to 5.6 were limed to pH 6.7 soil mineralised N doubled (Nyborg and Hoyt, 1978) providing increased concentrations of inorganic nitrogen for loss via nitrification and denitrification. The mole fraction of N<sub>2</sub>O and N<sub>2</sub> released is found to increase as the pH of soils decreases (Yamulki *et al.*, 1997). Smith and Chalk (1980) noted that immobilisation of nitrite and associated gaseous nitric and nitrous oxide evolution was inversely related to soil pH. In soils where denitrification is the dominant source of nitrogenous gases then N<sub>2</sub>O emissions tend to decrease with increasing pH in acid soils (pH below 5-6) (Granli and Bóckman, 1994) although the rate of denitrification has been shown to increase with increasing pH (optimum pH 7 – 8) (Conrad, 1995), this increase in rate and associated decrease in N<sub>2</sub>O production is due to a decrease in the N<sub>2</sub>O:N<sub>2</sub> product ratio of denitrification (Haynes, 1986).

The production of nitric oxide from acidic soils is sometimes greater than production from less acidic soils (Conrad, 1996) and the production of NO was significantly increased in incubation experiments with increasing soil sample size, N<sub>2</sub>O did not, this would indicate that NO production is restricted to micro sites in some soils.

#### 1.7.4. Soil and air temperature

Like most biological processes both nitrification and denitrification are affected by temperature as a result of Michaelis-Menten kinetics for enzyme catalysed reactions. As temperature increases then nitrification and denitrification rates will increase, this has implications for the amount of leakage from the model of Firestone and Davidson (1989). Yamaulki *et al.* (1997) found a significant positive correlation between soil temperature and nitric oxide emission rates, similar results were found by Clayton *et al.* (1997). Williams *et al.* (1987) noted a seasonal effect on field flux of NO which was correlated with temperature (soil and air), as temperature increased so did the flux of NO, this however would depend upon the dominant source of NO since nitrification and denitrification are predominantly aerobic and anaerobic processes respectively (Conrad, 1995).

#### 1.7.5. Microbial species diversity

Very little work has been done on the effects of species diversity and the nitrogen cycle, mainly due to the difficulties in determining true species diversity of environmental samples (Pankhurst *et al.*, 1996). However the importance of a diverse microbial community can be discussed in terms of general effects of biodiversity on ecosystems functioning. It is thought that more diverse microbial population will result in stabilised ecosystem functioning (Pankhurst *et al.*, 1996) and therefore species diversity will affect the production and emissions of NO and N<sub>2</sub>O.

The effects of land management treatments in the form of tillage and fertiliser applications on ammonia oxidiser diversity and nitrification rates showed no correlation between changes in nitrification rate and ammonia oxidiser community despite significant effects of treatment on nitrification rates DGGE 16S rDNA profiles did not indicate any change in the  $\beta$  – proteobacterial ammonia oxidisers (Phillips *et al.*, 2000). In another study Okano *et al.* (2004) used quantitative real time PCR of the *amoA* gene to quantify the effects of ammonium on population size of ammonia oxidising bacteria, they found that with increasing N addition the AOB

population density increased and net nitrification rates were also higher in N amended soils indicating a link between function and numbers of ammonia oxidisers.

Recently Gray *et al.* (2003) have measured changes in microbial communities and been able to link them with the effects of land management, they found that lime had the greatest influence on the AOB populations resulting in changes of the  $\beta$  – proteobacterial ammonia oxidisers and also increased nitrification potential, these effects however were masked over time by changes in soil conditions over the growing season. The emission of nitrous oxide with ammonium addition and changes in the ammonium oxidiser community by Avrahami *et al.* (2003) indicated increased nitrous oxide emissions were correlated with ammonium addition and nitrification but no detectable changes in the DGGE banding profiles of the *amoA* gene.

#### **1.7.6. Carbon availability (type and source)**

The microbial activity in soils is determined primarily by the soil carbon content as this provides both a source of energy and cellular carbon (Conrad, 1995). Studies of denitrification in permanent pasture soils showed that the availability of organic carbon in the form of glucose reduced the  $N_2O:N_2$  ratio as a result of denitrification (Dendooven *et al.*, 1996).

### **1.8. The use of stable isotopes**

Most elements of biological interest are present in two or more stable isotopic forms, one of which is usually present in far greater abundance than the other; in the case of nitrogen there are two stable isotopes,  $^{14}N$  (99.6337%) and  $^{15}N$  (0.3663%). The  $\delta^{15}N$  for atmospheric  $N_2O$  is 7 ‰ in pacific air (Kim & Craig, 1990) and varies greatly throughout the world depending upon the source of nitrogen and the biological processes involved in it's production (Webster & Hopkins, 1996).



### 1.8.1. Measurement of $\delta^{15}\text{N}$

The convention for expressing N isotope ratios is based on the international standard of atmospheric air (Mariotti, 1983) where the  $\delta^{15}\text{N}$  of  $\text{N}_2$  is 0‰. The ratio of heavy and light isotopes in a sample, compared to a standard, is measured using an isotope ratio mass spectrometer (IRMS). A gas chromatograph (GC) is usually in series with and upstream from the IRMS, these two combined allow the analyses of specific elements of a compound from a mixture. Until quite recently stable isotopes have been used at enriched levels where a known amount of nitrogen at approximately 99% atom %  $^{15}\text{N}$ , (i.e. enriched with the heavier isotope) has been used to determine the rates of nitrification and denitrification both in the field and in the lab (Murphy *et al.*, 1999). This “pulse” of heavy isotope can easily be detected and if specific compounds of nitrogen are used then specific functional metabolic pathways for specific groups of microorganisms can be isotopically traced.

The use of natural abundance versus enrichment approaches has advantages and disadvantages, at the natural abundance level the need for amendments with additional labelled substrate is not required, thus artificially high process rates as a result of increased substrate addition are not measured at natural abundance levels; however larger amounts of N for isotope analyses are required at natural abundance levels which makes quantification of trace gases and nutrients difficult under experimental conditions. The effects of fractionation are also best measured at natural abundance levels since often detection of minute changes in the heavy isotope are used to quantify discrimination of a process, with enrichment additions the fractionation is “swamped” by the amendment.

### 1.8.2. Factors affecting $\delta^{15}\text{N}$ isotope ratios in the nitrogen cycle

Stable isotopes have rapidly become a very useful tool for ecologists, enabling them to map the origins of materials in the environment as a result of the predictable physical and enzymatic based discrimination between biological and non-biological materials leading to different isotopic compositions (Ehleringer *et al.*, 1986). Isotopes because of their different masses undergo fractionation as a result of

their respective reaction rates ( $k$ ). The way in which this effects the isotopic composition of the reaction products is dependent upon the type of reaction and the enzymes, redox potentials and environmental conditions involved (Yoshida, 1988; Lajtha & Michener, 1994).

The isotopic fractionation of stable isotopes during chemical/biological reactions refers to the enrichment or depletion of the heavy isotope relative to the lighter isotope. Fractionation during unidirectional reactions can be expressed using Equation 4, where  $k_1$  and  $k_2$  are the reaction rates of the light and heavy isotopes respectively which is also known as kinetic fractionation.

Equation 4. Isotopic fractionation factors for unidirectional reactions

$$\alpha = k_1 / k_2$$

In a closed system the fractionation factor described in Equation 4. will vary as the reaction proceeds where substrate becomes limiting; however in non closed systems the fractionation factor will remain constant if substrate microbial process and populations are constant.

Under equilibrium reactions a larger activation energy is required to dissociate an isotopically heavy chemical species as opposed to a light one, therefore an isotopically light atom or ion will be bonded less strongly at equilibrium (Höberg, 1997) and can be expressed using Equation 5.

Equation 5. Isotopic fractionation factors for equilibrium reactions

$$\alpha = \delta_A / \delta_B$$

Ion exchange is another equilibrium reaction, which might involve isotope fractionation, the fractionation factors for various processes in the nitrogen cycle are shown in Table 1.

*Table 1. Fractionation factors ( $\alpha$ ) for processes in the nitrogen cycle due to kinetic fractionations (Höberg, 1997).*

Process	Fractionation factor ( $\alpha$ )
N mineralisation (org N to $\text{NH}_4^+$ )	$\approx 1.000$
$\text{NH}_3$ volatisation	1.029
Diffusion of $\text{NH}_4^+$ , $\text{NH}_3$ and $\text{NO}_3^-$ in soln.	$\approx 1.000$
Nitrification	1.015 – 1.035
Denitrification	1.000 – 1.033
N assimilation	1.000 – 1.020

Fractionation factors can be used to determine the isotope ratio of a substrate without actually having to measure it, the nitrification of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  has large effects on the isotope composition whereas the second step of nitrification which is not rate limiting should not lead to further fractionation. The isotope effects during nitrification have only been studied for *Nitrosomonas europaea* and not for organisms other than *Nitrosomonas* and heterotrophic nitrifiers, which could vary considerably in terms of fractionation (Höberg, 1997).

### 1.8.3. $^{15}\text{N}$ pool dilution and enrichment

Until recently no research groups using  $^{15}\text{N}$  labelling of soil ammonium pools for determining the gross rates of N-mineralisation and hence pool dilution had looked at the effects of their methods of labelling soils on the gross N-mineralisation. This has very important consequences for much of the work done on  $^{15}\text{N}$  pool

dilution prior to that of Murphy *et al.* (1999), who reported that not only is the method of application important but also the soil type when trying to achieve a uniform labelling of the soil ammonium pool. Addition of  $^{15}\text{NH}_3$ -air gas mixture to a headspace volume compared with injection of  $^{15}\text{NH}_3$ -air gas mixture into re-packed soil cores resulted in lower calculated rates of gross N-mineralisation and  $\text{NH}_4^+$  consumption. The  $^{15}\text{N}$  pool dilution technique makes it possible to determine the input and output flow into and out of a labelled pool by determining changes in the total pool size and in  $^{15}\text{N}$  enrichment in that pool during an incubation experiment (Tietema and Wessel, 1992).

Gross rates of nitrification determined using  $^{15}\text{N}$  pool dilution using an injection technique with ammonium sulphate and potassium nitrate ranged between 12 and 46% of the gross mineralisation rates, the use of two substrates allowed the identification of the dominant microbial processes, by looking at the gross rates of nitrification and denitrification (Davidson *et al.*, 1990). Rates of nitrification and denitrification determined by pool dilution techniques are gross "potential" rates and not actual rates which are net rates of production and consumption of either nitrification or denitrification.

Pool dilution techniques on their own provide information about nitrogen cycling within soils as a whole, but when specific inhibitors of nitrification and denitrification are used in conjunction with the pool dilution techniques then the resolution of microbial processes is increased further.

#### 1.8.4. Natural abundance $^{15}\text{N}$

The use of natural abundance isotopes is perhaps one of the most recent advances in ecology due to rapid development of GC-IRMS for analysis of low concentrations of trace gases in the atmosphere. Natural abundance of isotopes in the nitrogen cycle is perhaps of greatest importance. It is difficult to determine sources of NO and  $\text{N}_2\text{O}$  in the field without using specific inhibitors and researchers are now using natural abundance isotope ratios for these two gases to determine their source. Fractionation during nitrification and denitrification has been found to result in  $^{15}\text{N}$



enrichment of the soil N pool, and leaching or gaseous loss of the lighter  $^{14}\text{N}$  (Emmett *et al.*, 1998). Nitrification has also been found to discriminate against  $^{15}\text{N}$  in the substrate more than does N mineralisation, hence  $\text{NH}_4^+$  can become isotopically heavier than the organic N from which it is derived (Höberg, 1997).

Webster and Hopkins (1996) utilised both nitrogen and oxygen stable isotope signals to determine the source of  $\text{N}_2\text{O}$  from soils and pure cultures under different soil water potentials, whilst this did indicate the source of  $\text{N}_2\text{O}$  with regards to either nitrification or denitrification it was not conclusive due to the shifts in signals of two different soils incubated at the same time under the same conditions.

The natural abundance signals of  $^{15}\text{N}$  for various nitrogen species have been documented previously, some of the more relevant ones are shown in Figure 11., the isotopic composition of the product is related to that of the substrate and process which formed the product, as is the case with  $\text{NO}_x$  derived as a result of combustion (-15‰) and clean air (-10‰)

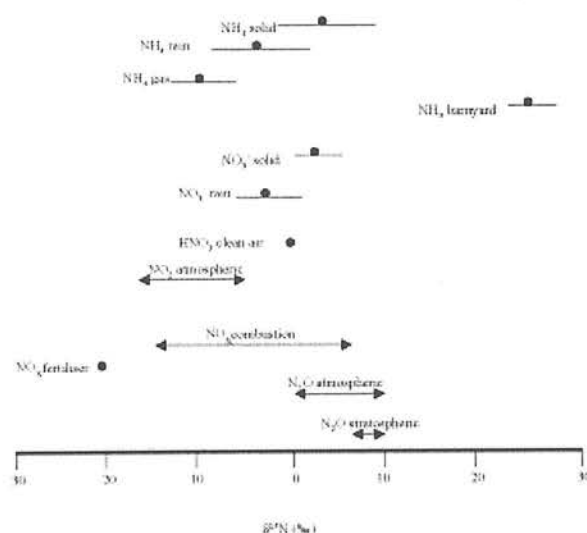


Figure 11. Summary of  $\delta^{15}\text{N}$  measurements of various nitrogen species (Trumbore, 1995 and Macko & Ostrom, 1994)

The spatial variability in  $\delta^{15}\text{N}$  can be considerable, Sutherland *et al.* (1991) determined the variability in plants and soils at two different scales and noted that at the larger scale (110 x 110 m)  $\delta^{15}\text{N}$  values were high and were associated with depressions in the soil, where denitrification rates were high.

Both N and O isotope ratios can be used to determine the source of nitrate, plant N increased at high rates of N addition which could result from fractionation due to N losses via nitrification and denitrification, hence the isotopic composition of NO and N<sub>2</sub>O could provide a useful tool for identifying the dominant processes in the field. The isotope signal of  $\delta^{18}\text{O}$  for NO<sub>3</sub><sup>-</sup> can differ by about 50 ‰ between nitrate deposited as fertiliser-N and that produced by nitrification, nitrate derived from heterotrophic nitrification may also be very different isotopically due to the oxygen source being that of CO<sub>2</sub> as opposed to H<sub>2</sub>O.

## Chapter Two. General Methods

### 2.1. Field site description

The experimental field site is an upland grassland, the site is 320 m above sea level and 55°28'N/2°14'W (Plate 1), the major soil sub-group is a brown forest soil (%N 0.56, %C 7.61, pH (H<sub>2</sub>O) 4.54-4.81, pH (CaCl<sub>2</sub>) 4.04-3.88) and forms part of the Sourhope series. A typical soil profile is show in Plate 2.



*Plate 1. Sourhope Rigg Foot Soil Biodiversity site from Block 1A*



*Plate 2. Soil profile from Sourhope control plots 0-10 cm depth.*

The experimental structure was that of a randomised block design with five treatments; two controls, nitrogen ( $256 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  as  $\text{NH}_4\text{NO}_3$ ), lime ( $\text{CaCO}_3$   $6000 \text{ kg ha}^{-1} \text{ yr}^{-1}$ ), nitrogen and lime, shown in Figure 12. Each main plot was split into a sub-plot design (Figure 13).

Gas flux chamber placement at the field site was permanent and careful consideration was taken when placing chambers. Each of the general sampling areas S,T,U and V within the treatment sub-plots were assigned a random number, for each replicate of the following treatments; control 1, nitrogen, lime and nitrogen plus lime. The chambers were then placed on the allocated sampling site for this project within the general sampling area S,T,U or V with the lowest random number.

Running parallel down the field site from top to bottom were ridges and furrows, these often bisected the general sampling areas S,T,U and V. Therefore it was necessary to allocate chambers only to ridges due to obvious soil moisture differences between ridges and furrows where water tended to collect.

The chambers remained in situ throughout the experiment and were removed during mowing events and then replaced. The semi-permanent deployment of chambers reduced the need for deployment days before a flux measurement to prevent disturbance and increased nitric and nitrous oxide flux. In summary the chambers were placed within the lowest numbered general sampling area, which did not have a furrow preventing chamber placement.



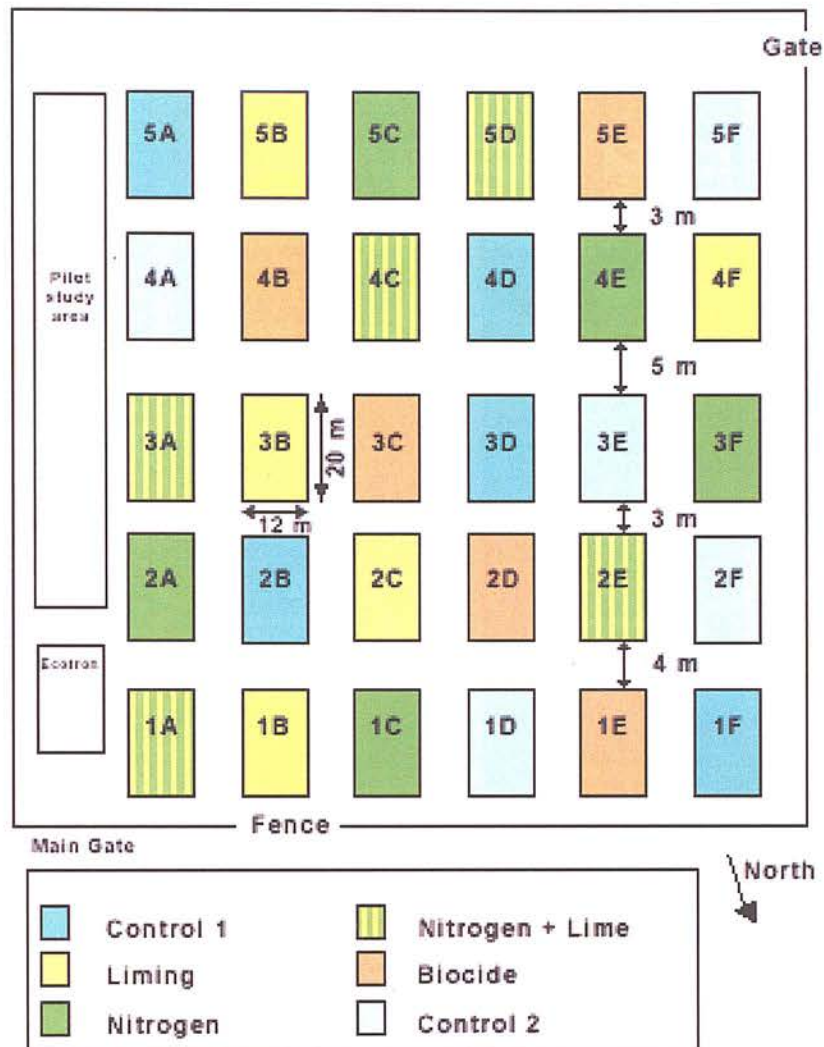


Figure 12. Sourhope randomised block treatment allocations

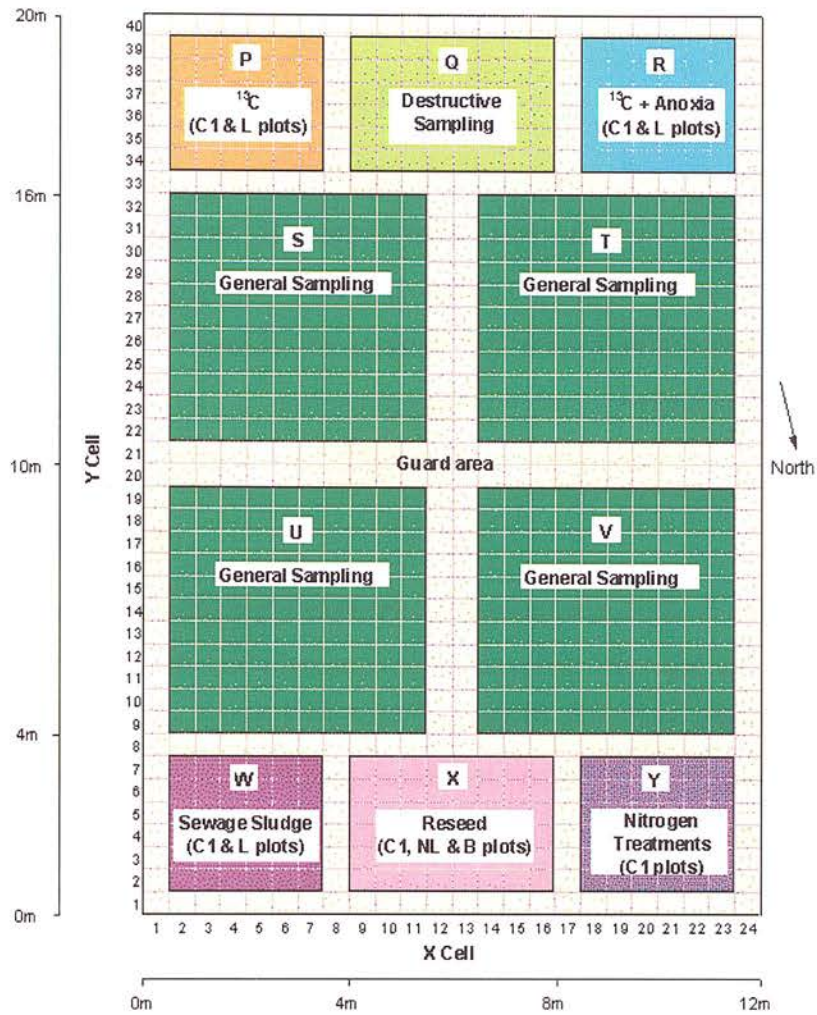


Figure 13. Sourhope sub-plot design

The treatments were applied on a yearly basis and the field site mowed using a small sit-on mower, all grass cuttings were removed from the site to simulate the effects of grazing. The mowing of the field and treatment additions covering the field seasons from 1999 to 2002 are shown in Table 2.

## 2.2. The measurement of trace gas fluxes

In this thesis all units of field flux measurements, the gas flux density or net rate of gas exchange per unit of cross-sectional surface area will be presented as  $\mu\text{g m}^{-2} \text{h}^{-1}$ . This is defined as positive for a net emission of gas whilst a negative value is net uptake of the gas of interest. The units of flux for soil microcosm studies will be presented as  $\mu\text{g g}^{-1} (\text{dry wt.}) \text{h}^{-1}$  following gas flow rate experiments (Chapter 3).

## 2.3. Chamber design

The chambers used for fieldwork measurements of both nitric and nitrous oxide consisted of a 40 cm diameter polypropylene tube cut to 20 cm lengths. Flanges made from polyvinylchloride (4.5 cm width) were then attached to the top of the chambers and sealed with silicone sealant at the joint. A gas sample tube made from nylon tubing that protruded approximately 15 cm into the centre of the chamber was sealed through the wall of the chamber using silicon sealant. At the sample end of this tubing a three way PTFE tap (Sterilin) was attached using silicone rubber tubing (Figure 14).

Table 2. Treatment application dates and mowing schedule at the Sourhope Rigg Foot field site

Lime	CaCO <sub>3</sub> 6000 kg ha <sup>-1</sup> yr <sup>-1</sup>		Mowing		Nitrogen		256 kg N ha <sup>-1</sup> yr <sup>-1</sup> as NH <sub>4</sub> NO <sub>3</sub>	
	Date	Blocks	Date	Blocks	Date	Blocks	Date	Blocks
	07/05/1999	5B, 5D	01/06/99-09/06/99	blocks 1-5	02/05/1999	4C, 4E, 5C, 5D		
	15/05/1999	4C, 4F	29/06/99-05/07/99	blocks 1-5	03/05/1999	1A, 1C, 2A, 2E, 3A, 3F		
	17/05/1999	2C, 2E, 3A, 3B	26/07/99-29/07/99	blocks 1-5	28/09/1999	4C, 4E, 5C, 5D		
	19/05/1999	1A	23/08/99-26/08/99	blocks 1-5	29/09/1999	1A, 1C, 2A, 2E, 3A, 3F		
	20/05/1999	1B	21/09/99-25/09/99	blocks 1-5	17/04/2000	4C, 4E, 5C, 5D		
	05/04/2000	5B, 5D	08/05/00-11/05/00	blocks 1-5	18/04/2000	1A, 1C, 2A, 2E, 3A, 3F		
	06/04/2000	3A, 3B, 4C, 4F	07/06/2000	blocks 1-5	16/05/2000	1A, 1C, 2A, 2E, 3A, 3F, 4C, 4E, 5C, 5D		
	07/04/2000	2C, 2E	05/07/2000	blocks 1-5	11/04/2001	3A, 3F, 4C, 4E, 5C, 5D		
	10/04/2000	1A, 1B	03/08/2000	blocks 1-5	12/04/2001	1A, 1C, 2A, 2E		
	20/04/2001	5B, 5D	30/08/2000	blocks 1-5	23/05/2001	1A, 1C		
	21/04/2001	4C, 4F	07/05/01-18/05/01	blocks 1-5	24/05/2001	2A, 2E, 3A, 3F		
	24/04/2001	3A, 3B	04/06/01-08/06/01	blocks 1-5	25/05/2001	4C, 4E, 5C, 5D		
	25/04/2001	2C, 2E						
	26/04/2001	1A, 1B						

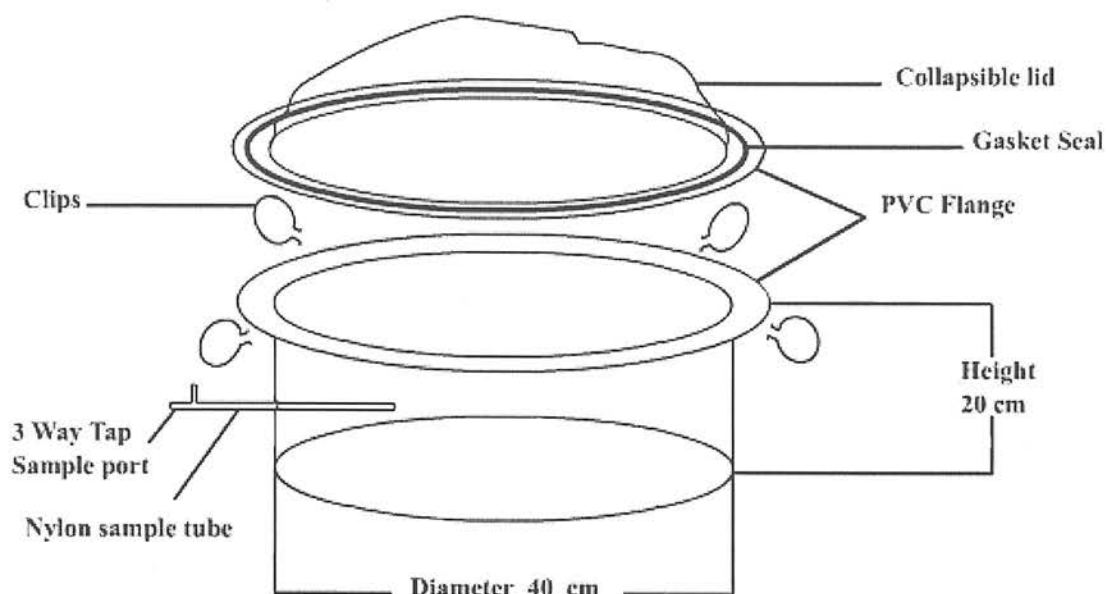


Figure 14. Static non-vented chamber used for field gas measurements

The lids for the chambers consisted of identical flanges used on the chamber body with a ring of rubber draft excluder attached on the under side created a gas tight seal. The collapsible lids consisted of UV permeable plastic sheeting that was attached to the flange using Duct tape (3M, Canada) as shown in Plate 3. The volume of each lid was then noted and assigned a lid number.

## 2.4. Methods of analysis of nitric oxides

Nitric oxide measurements were made using a ThermoOnix 42 CTL NO<sub>x</sub> analyser. This method relies upon the principle of chemiluminescence, and in particular the reaction of NO and ozone shown in Equation 6.

Equation 6. Nitric oxide reactions with ozone







*Plate 3. Field chamber and sampling for nitrous oxide using a 500 ml Hamilton syringe and 1 L Tedlar bag.*

Ozone that is generated reacts with NO to produce a luminescence at a wavelength between 600 and 300 nm, with an intensity that is linearly proportional to the concentration of NO. Air is drawn into the analyzer at a flow rate of approximately  $1.5 \text{ l min}^{-1}$  and passed through an ozonator, which generates air with excess ozone; this is then passed through a reaction chamber.

Sample air is drawn through a filter and capillary tube then passed through one of two flow paths; either directly to the reaction chamber where the sample contains both NO and NO<sub>2</sub> where the NO reacts with ozone and the light intensity is measured using a photo multiplier tube (PMT), this is a measure of the NO concentration only.

Sample air is then switched to a second flow path in which  $\text{NO}_2$  is transformed to  $\text{NO}$  with molybdenum  $\text{NO}_2$  to  $\text{NO}$  converter ( $325^\circ\text{C}$ ). This air is then passed through to the reaction chamber and the light intensity measured which gives the combined  $\text{NO}$  and  $\text{NO}_2$  concentration.

The  $\text{NO}_2$  concentration is then calculated from the difference in  $\text{NO}$  concentration between the two flow paths of  $\text{NO}$  and  $\text{NO} + \text{NO}_2$  concentration.

The flow rates through both flow paths are controlled using a capillary tube and monitored using a flow sensor, this is essential for precise computation of the  $\text{NO}$  concentration.

## 2.5. Methods of analysis of nitrous oxide

Nitrous oxide ( $\text{N}_2\text{O}$ ) was determined from samples collected in either Tedlar bags or 10ml syringes using gas chromatography, the field chambers had previously been tested for gas tightness in previous studies to determine nitrous oxide flux (Skiba *et al.*, 1990) it is also assumed that the net rate of trace gas exchange is constant over the sampling period (Livingston & Hutchinson, 1995), capping times of 30 minutes and one hour were tested for nitrous oxide flux, with a linear increase in headspace concentration over the capping times. Chambers were capped for 60 minutes due to the very low fluxes of nitrous oxide in early field measurements made in control plots.

The GC system consisted of a Chrompak CP 9000 with a Poropak Q column (id.0.53 mm, length 12.5 m, o.d. 0.70 mm) fitted with an ECD and a 10 port sample and injection valve as shown in Figure 15. The oven temperature was set to  $30^\circ\text{C}$  and the ECD detector to  $350^\circ\text{C}$ . The flow through the separation column was controlled using a small needle valve and set to  $5 \text{ ml min}^{-1}$ ; this gives a retention time of approximately 2.87 minutes for  $\text{N}_2\text{O}$ . The sample valve was used to remove compounds that could interfere with the ECD; mainly  $\text{H}_2\text{O}$  that could carry over to subsequent samples.

In the load position the sample is injected into the sample loop (0.5ml) via an injection port sealed with a rubber septa. The valve is then switched to the inject

position and the N<sub>2</sub> carrier gas carries the sample in the sample loop into the pre-column and through the separation column in the GC. After 55 seconds the valve is switched back to inject and the slower moving gases and water are back flushed with nitrogen through the pre-column to vent.

Calibration gas standards (typically 1ppm N<sub>2</sub>O in 80% N<sub>2</sub> and 20% air) were injected at the beginning and end of the gas analysis to account for any drift in the GC and also as a standard for calculation of N<sub>2</sub>O concentrations from mV to ppm (parts per million) of the unknown gas samples. A calibration curve for nitrous oxide was determined periodically and the response of the GC ECD was linear between 0.31 and 2 ppm N<sub>2</sub>O (Peak area (mV) = 10279 \* N<sub>2</sub>O ppm + 37777, R<sup>2</sup> = 0.9207 P < 0.01) and 2 ppm and 11 ppm N<sub>2</sub>O (Peak area (mV) = 144769 \* N<sub>2</sub>O ppm – 201513, R<sup>2</sup> = 0.9968 P < 0.05).

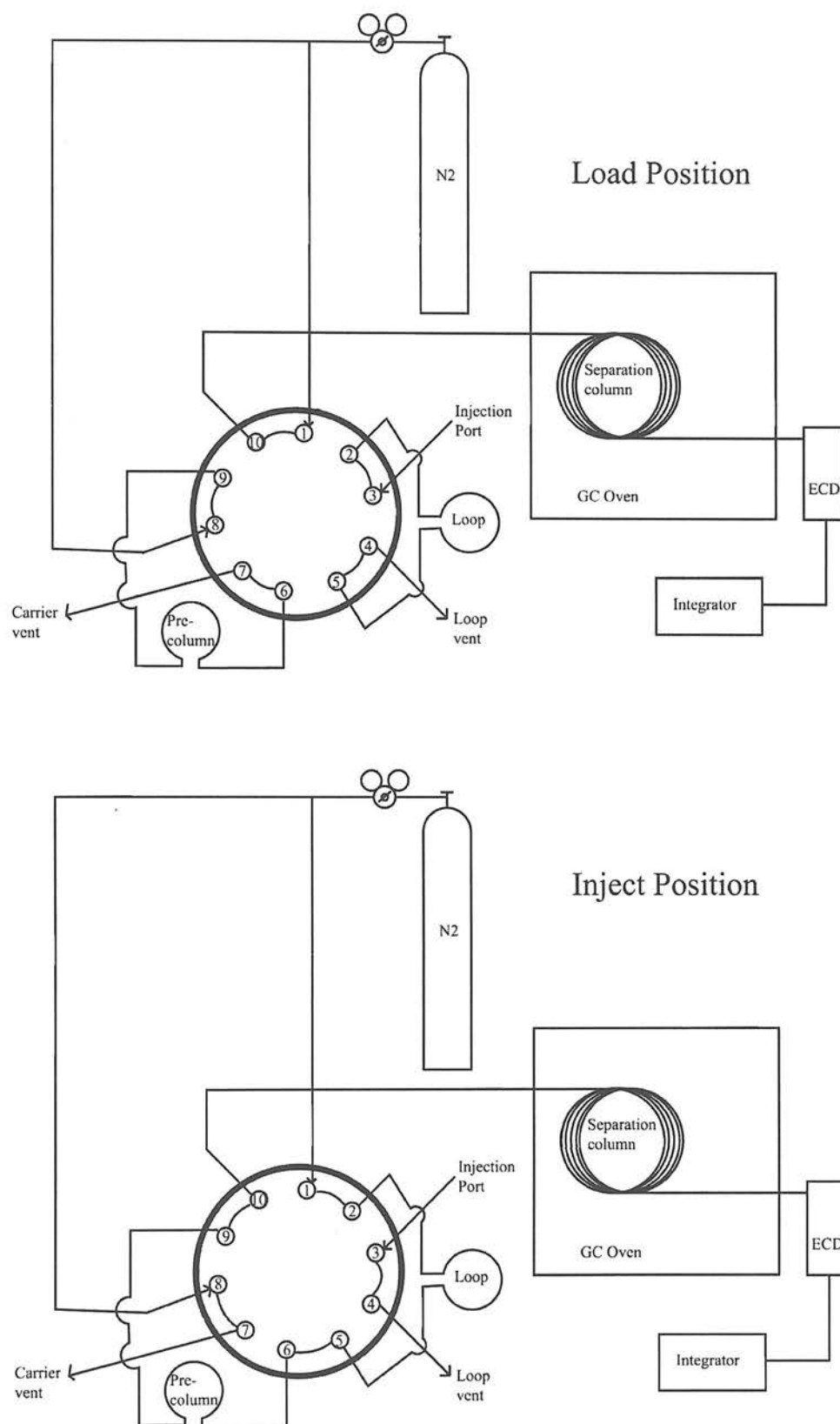


Figure 15. Schematic diagram of Chrompak GC and backflush valve system

## 2.6. The measurement of trace gas fluxes in soil microcosm experiments

In all soil microcosm experiments acrylic plastic tubes were cut into 20 cm lengths, the ends of which were sealed using plastic lids from 120 ml specimen cups. Bicycle tyre inner tubes cut into two inch rings formed a gas tight seal between the lid and microcosm, the exact details of soil microcosms are shown in Figure 16.

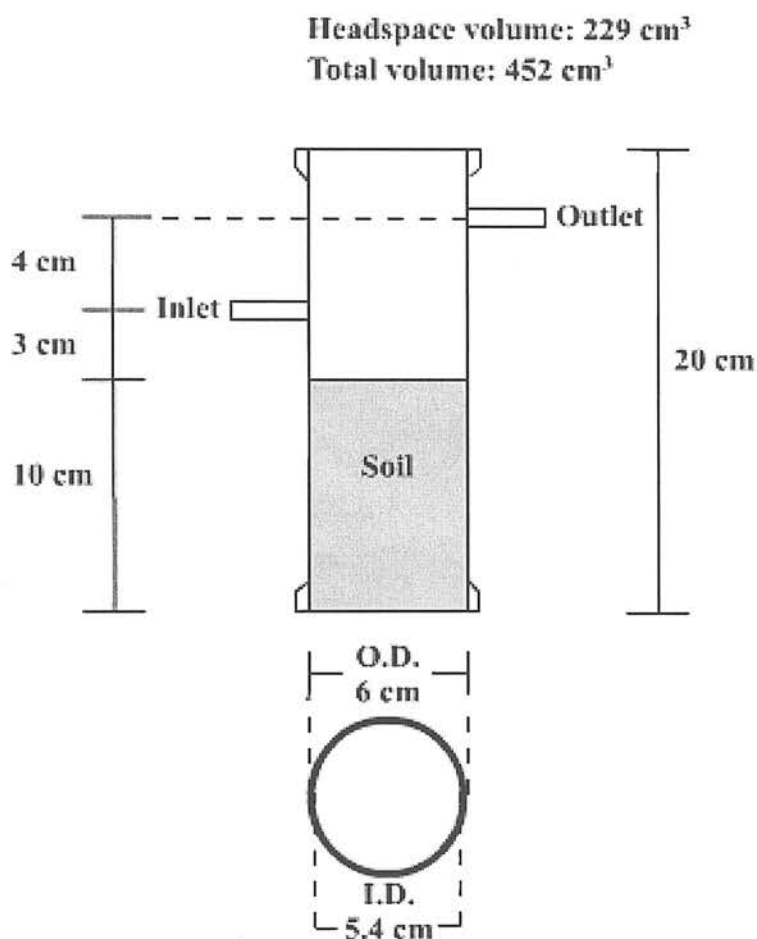


Figure 16. Soil microcosm design

Sample ports were drilled at 13 and 17 cm from the column base, rubber grommets were then inserted to provide a gas tight seal between the soil microcosms and the PTFE tubing of the gas measurement system.



The following column methods were used to determine gaseous fluxes; for nitric oxide measurements steady state vented type chambers were used as shown in figure 18. Nitrous oxide measurements were made using a non-vented non steady state system where three way taps and PTFE tubing were inserted into the rubber grommets and the concentration of nitrous oxide allowed accumulate in the headspace over a period of time.

With the steady state vented system (Figure 17 and Plate 4) used for nitric oxide measurements the flow rate of air through the soil column was controlled using a mass flow controller (Aera 770C), set at a constant flow rate of  $40 \text{ ml min}^{-1}$ . A second mass flow controller (Tylan) was used to dilute the column air with further  $\text{NO}_x$  free air from the lab which was scrubbed with a Purafil® and charcoal air filter.

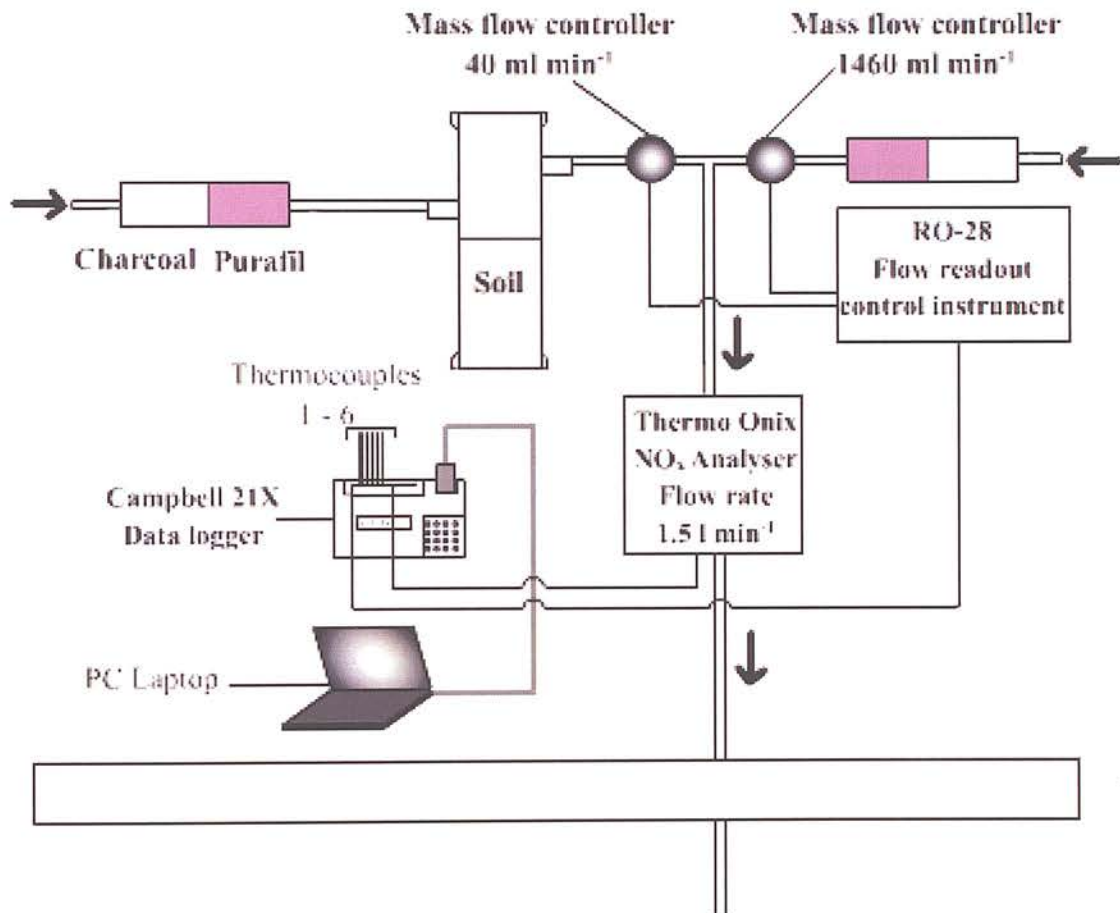
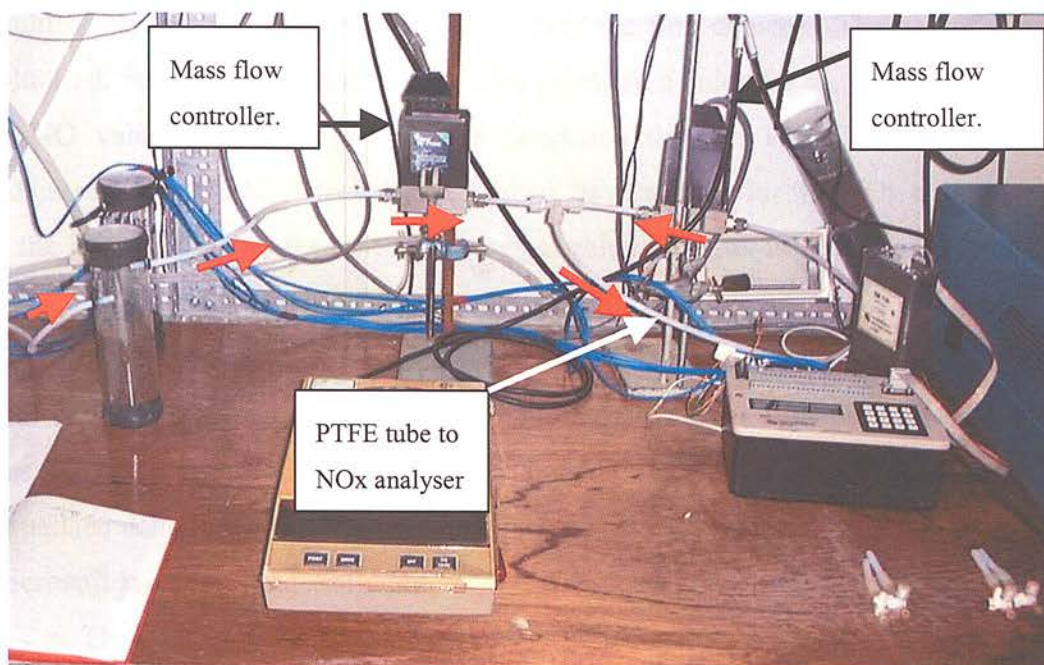


Figure 17. Schematic diagram of  $\text{NO}$  flux measurement system for soil microcosm experiments, arrows indicate the flow of lab air.



*Plate 4. NO flux measurement system for soil microcosm experiments, mass flow controllers are indicated by black arrows with direction of air flow with red arrows.*

Both flow controllers were controlled using a Tylan RO-28 mass flow controller and the flow rate from both mass flow controllers was logged every 10 seconds using a Campbell 21X data logger.

The flux density of nitric oxide, determined using the steady state system in Figure 17, was modeled using Equation 7; where  $s$  is the flow rate through the soil column ( $\text{l min}^{-1}$ ),  $A$  is the chamber basal area ( $\text{m}^2$ ) or soil dry weight (g) and  $C_o$  and  $C_i$  the concentration of nitric oxide (ppm) in the outgoing and incoming air respectively,  $M$  is the molar gas volume (22.4 at S.T.P.) and 14 the grams of N in one mole of NO.

Equation 7. Flux density calculations for nitric oxide emissions

$$f = \frac{s}{A} (C_o - C_i) \times M \times 14$$

An initial blank microcosm without soil was placed in line with the measurement system so that any reactions with  $\text{NO}_x$ , ozone and the microcosm walls could be taken into account when calculating the flux density. The use of a blank column at the start and end of the run also provided a value for  $C_0$  in Equation 7 with an  $\text{NO}$  value of zero following the flowpaths through Purafil and charcoal as indicated in Figure 17. This zero  $\text{NO}_x$  blank gave a value for the  $\text{NO}_x$  concentration in the incoming air to the soil microcosms which could not be measured using this set up.

Once the baseline zero air blank was stable, a soil column was placed inline with the  $\text{NO}_x$  analyser and left for a minimum of 15 minutes, or until an equilibrium was reached for the  $\text{NO}$  and  $\text{NO}_2$  gas exchange within the soil microcosm. This was visualised using a lap top computer connected to the Campbell 21X data logger using Microsoft Hyperterminal software.

The soil microcosm was then removed and the  $\text{NO}_x$  concentration allowed to reach the baseline level (approximately 2 minutes) before the next soil microcosm was analysed.

## 2.7. Measurement of nitrous oxide

Nitrous oxide measurements were made using the soil microcosm shown in Figure 16 under non-steady state, non-vented conditions in which a small length of PTFE tubing with a three way tap was inserted into the rubber grommets.

A 10 ml gas sample was taken from above the soil microcosm headspace prior to capping using a 10 ml plastic syringe; this was the time zero ( $T_0$ ) gas sample. The soil microcosm was then capped and the three way taps with PTFE tubing positioned so that the soil microcosm was closed to the lab air. After 15 minutes another gas sample was taken using a 10 ml syringe, this was filled with lab air (ambient  $\text{N}_2\text{O}$  concentration) prior to sampling and then flushed into and out of the soil microcosm headspace three times prior to taking another 10 ml gas sample.

All gas samples were then analysed using the GC system described previously, two five ml injections were made per syringe sample to validate the  $\text{N}_2\text{O}$  concentration/syringe. Nitrous oxide fluxes were determined using Equation 8, where

V is the enclosure volume ( $\text{m}^3$ ), A the basal area ( $\text{m}^2$ ),  $C_0$  and  $C_i$  the initial and end concentrations of nitrous oxide (ppb) measured over incubation time  $dt$  (hours), M is the molar gas volume (22.4 at S.T.P.) and 28 the grams of N in one mole of  $\text{N}_2\text{O}$ .

Equation 8. Flux density calculations for nitrous oxide emissions

$$f = \frac{V}{A} \times \frac{(C_o - C_i) \times M \times 28}{dt}$$

## 2.8. Soil inorganic nitrogen analysis

The inorganic N from soil samples was determined following KCl extraction, the use of KCl allowed the exchange of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  ions bound to clay particles with  $\text{K}^+$  and  $\text{Cl}^-$  ions respectively, thus giving available soil ammonium and nitrate concentrations (Rowell, 1994).

All glassware and plastic was thoroughly washed with Decon and rinsed repeatedly with de-ionised water prior to use.

The soil (20g wet weight) was weighed out into small plastic pots and 100 ml of 1M KCl was then dispensed into the pot (Jencons, Zippette) and then shaken using an orbital shaker at 280 rpm (Gallenkamp) for one hour.

The soil KCl extracts were then filtered through a Whatman No. 42 filter paper using a polypropylene funnel into a polypropylene sample pot. The filtrate was then shaken and a sub sample stored in a 30ml glass vial for up to 2 weeks at  $5^\circ\text{C}$  prior to colorimetric analysis.

The KCl extracts were analysed using a continuous flow system (Chemlab Instruments Ltd) where sample is drawn through Tygon tubing using a peristaltic pump and mixed with reagents in coiled glass tubing. Air bubbles are introduced with equal spacing to aid sample reagent mixing and maintain spacing between consecutive samples.



The nitrate determination involved reduction of nitrate to nitrite using a hydrazine copper reagent after which nitrite — N was determined colorimetrically after formation of an azo dye at a wavelength of 540 nm. The ammonium colour development involved complexing of ammonium with indophenol blue which was then measured using a colorimeter at a wavelength of 680 nm.

The absorbance of light is linearly proportional to the concentration of ammonium and nitrate N up to 2.5 ppm ( $\text{NH}_4$  - N and  $\text{NO}_3$  - N), samples were preceded by 6 standards at the start and end of a run and then every 12 samples by another standard selected at random with blank KCl solutions prior to and after a standard to prevent carry over to the next sample.

## 2.9. $^{15}\text{N}$ stable isotope pool dilution

The  $^{15}\text{N}$  pool dilution methods used in this study involved the addition of an isotopically “heavy” / “enriched”  $^{15}\text{N}$  nitrate source as  $\text{NH}_4^{15}\text{NO}_3$  at 99 atom %  $^{15}\text{N}$  to soil columns. These were then incubated for 3 days at constant temperature and moisture content maintained gravimetrically by addition of de-ionised water (Brooks *et al.*, 1989).

Either a destructive soil sample was taken or a soil sub-sample (25g wet weight) and the inorganic N extracted in 100 ml of 1M KCl using an orbital shaker (280 rpm) for one hour. Extracts were then filtered (Whatman No. 42 filter paper) and stored at 5°C for a maximum of 2 days prior to  $^{15}\text{N}$  extraction.

There are various methods for extraction of  $^{15}\text{N}$  labelled ammonium and nitrate from soil extracts and include steam distillation and methods developed for  $^{15}\text{N}$  extraction from water samples using dye complexes (Preston *et al.*, 1992).

The method of  $^{15}\text{N}$  extraction of ammonium and nitrate from soil KCl extracts used in these studies is a combination of that of Brooks *et al.* (1989) and Stark and Hart (1996) with a modified method for trapping the acidified discs between sheets of PTFE. Whatman GF/C filter discs (7cm diam) were used to give discs 7mm in diameter, these were acid washed with 2M HCl (4 x 50 ml washes) in a



Buchner funnel followed by washing with de-ionised water (6 x 50 ml) and then left to dry over night at 55°C.

Filter discs were acidified using 2.5 M  $\text{KHSO}_4$  (10  $\mu\text{l}$ ). The acidified filter discs were then sandwiched between two strips of PTFE using the aluminium block shown in Figure 18. and clamped together in a small vice to create a seal around the discs.

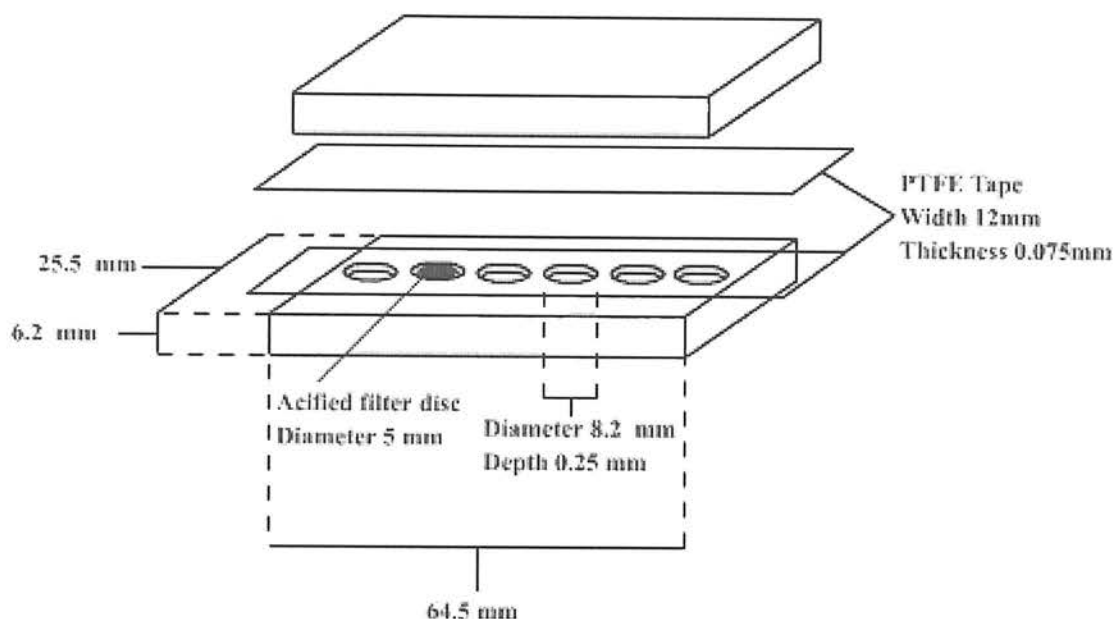


Figure 18. Aluminium block for making PTFE acid traps

The PTFE acid traps for  $^{15}\text{NH}_4$  extraction were then added to the extract immediately after adding the magnesium oxide (0.2 g) shown in Figure 19. These were then shaken for 3 days 20°C on an orbital shaker at 200 rpm. The PTFE strip was then removed from the extract solution using forceps and rinsed with de-ionised water before drying in a desiccator (4 hours) containing conc. sulphuric acid.

Devarda's alloy was then added (0.4 g) to the extract solution and another two PTFE acid traps added for extraction of  $^{15}\text{NO}_3$ , these were then shaken for

another 3 days at 20°C prior to removal of the acid traps and rinsing and drying as before.

The PTFE traps once dried were stored in sealed bags and then in larger plastic bags containing acid coated blotting paper to capture any atmospheric ammonia.

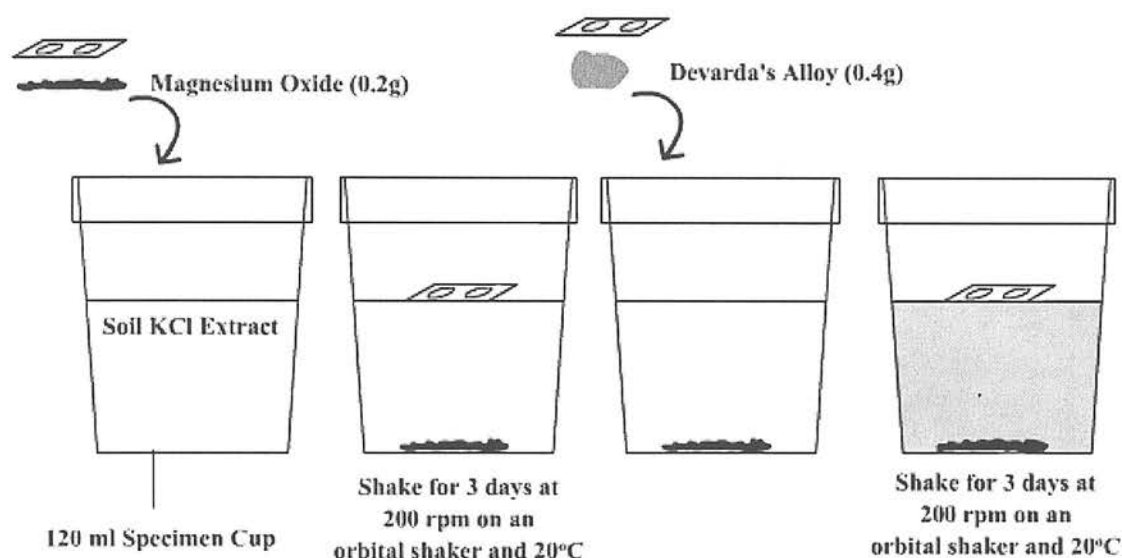


Figure 19.  $^{15}\text{N}$  extraction method.

The PTFE acid traps were then stored in a desiccator with concentrated sulphuric acid for up to three months, without any effects on the isotopic composition of the N on the discs (personal comm. Stark, 2001).

Samples were then sent for analysis at the NERC  $^{15}\text{N}$  Stable Isotope Facility, CEH Merlewood for analysis using a CF Isotope Ratio Mass Spectrophotometer and therefore placement directly into tin cups in this case was not possible.

## Chapter Three. Method Development and Evaluation

### 3.1. Evaluation of NO<sub>x</sub> reactions within a PTFE chamber

The use of a blank chamber to evaluate NO reactions with ozone and the chamber walls involved sealing the base of one of the field chambers with a sheet of PTFE (polytetrafluoroethylene). This chamber was then sealed using a collapsible lid following NO analysis with the Thermo Onix chemiluminescence NO analyzer (42TL).

The chamber was sealed for 15 minutes before measuring the concentration of NO within the chamber using the methods previously described in Chapter 2. The ambient ozone concentrations were measured at the CEH Edinburgh ozone monitoring station, the results of which are presented in Figure 20.

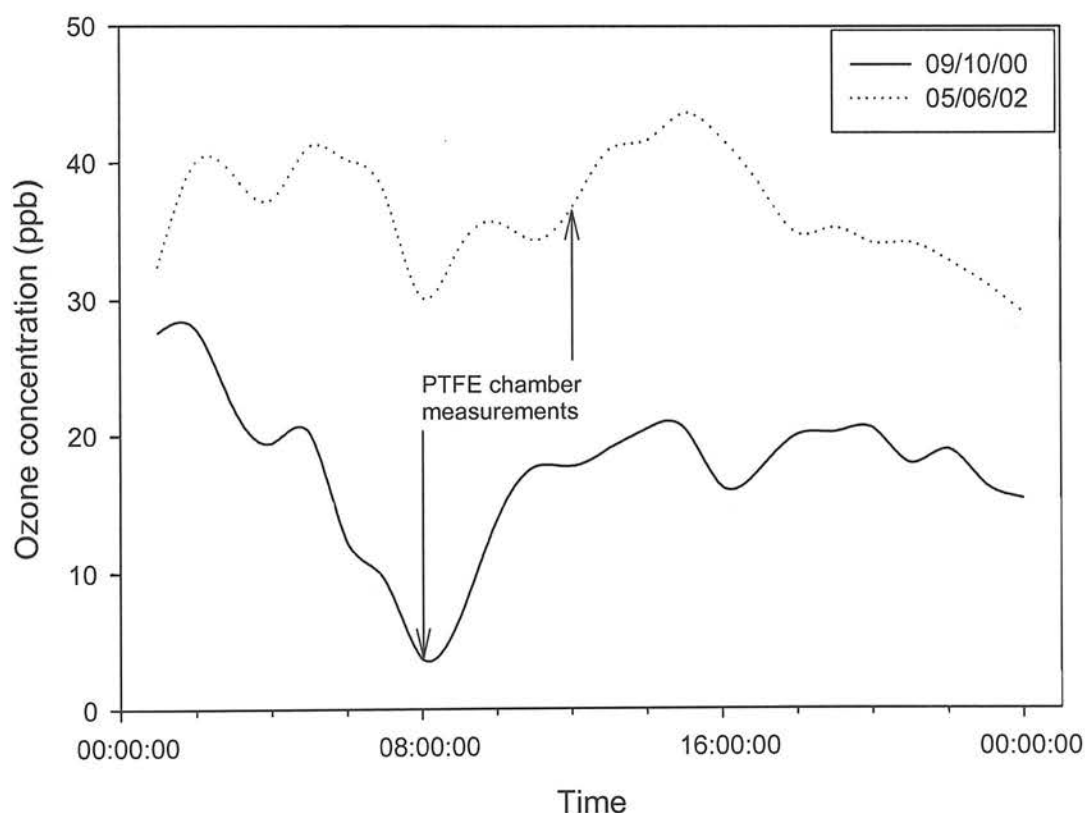


Figure 20. Ambient ozone concentration measured at the CEH Edinburgh Bush Estate constant monitoring station for the two PTFE chamber measurement days.

The PTFE chamber measurements were made on two days when ozone concentrations were low (09/10/00) and high (05/06/02). The concentration of NO decreased during the chamber capping time and was greater when ambient ozone concentrations were at their lowest and ambient NO concentrations were between 8 and 9 ppb (Figures 20. and 21.). The NO mixing ratio corresponds to the ratio of the volume of NO to the volume of the air which it is in, where 1 ppm ( $10^6$ ) is  $1\text{cm}^3$  of NO per  $10^6\text{ cm}^3$  of air. The units of ppb ( $10^9$ ) assume ideal gas behaviour and therefore concentration is not affected by temperature and pressure as they affect both the NO and the air which it is in to the same extent.

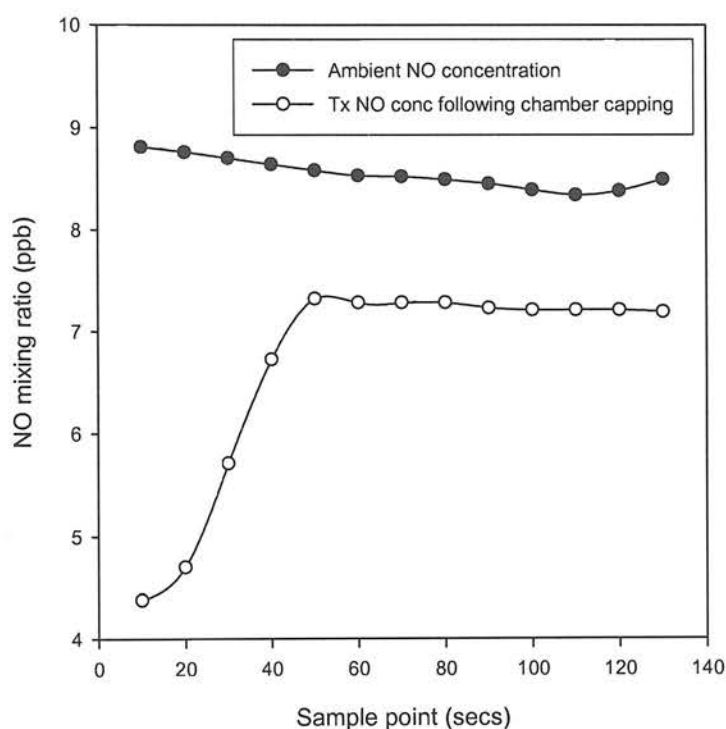


Figure 21. NO reactions with ozone and chamber walls within a PTFE chamber at low ambient ozone concentrations (09/10/00).

This difference of 1.2 ppb NO at ambient concentrations within the PTFE chamber (Figure 21.) represents loss of NO via reactions with ozone (Fowler *et al.*, 1998) and the chamber walls within the PTFE chamber.

The exact kinetics and proportion of NO oxidized by ozone and that lost to chamber walls cannot be determined from these experiments. However it does highlight the importance of ambient ozone concentration effects on NO flux dynamics. During the sample day with low ozone concentrations there were larger ambient NO concentrations, and there were losses of approximately 14% of the initial ambient NO concentrations via oxidation and deposition to chamber walls (Figure 22).

At lower ambient NO concentrations which were 7% of those measured on the lower ozone sample day (Figures 21. and 22.) ozone concentrations were much greater (35 ppb) as apposed to 5 ppb on the days where NO concentrations were greatest.

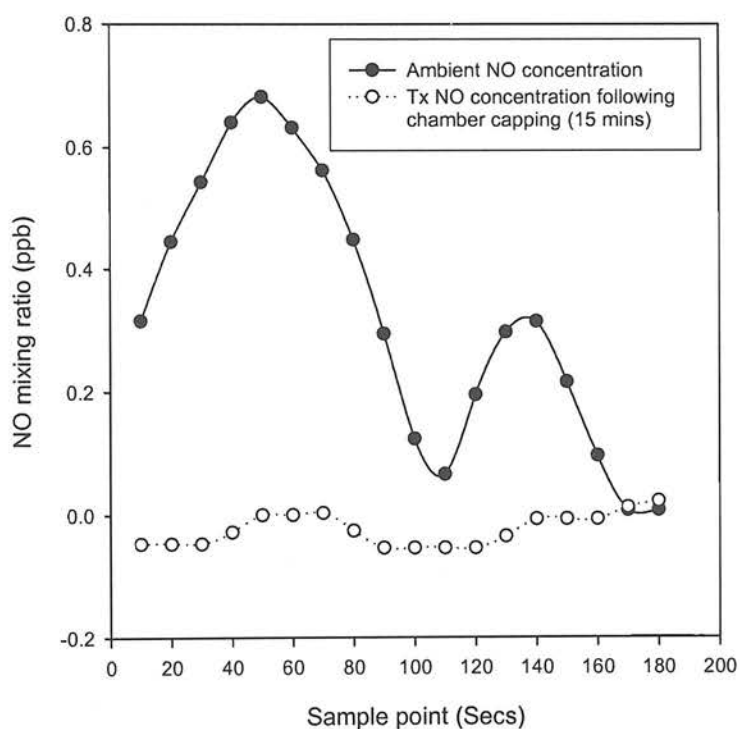


Figure 22. NO reactions with ozone and chamber walls within a PTFE chamber at high ambient ozone concentrations (05/06/02).



The decrease in NO concentration within the PTFE chamber was much greater as a percentage of the initial ambient NO concentration when ambient ozone concentrations were high, with a decrease of 0.7 ppb with initial NO concentration being 0.68 ppb. It would be reasonable to assume that low ambient NO concentrations on this day are a direct result of reactions with ozone which was at high ambient concentrations, and that NO compensation points for emission and deposition are significantly affected by the ambient ozone concentrations.

This loss via ozone and NO reactions can be accounted for in subsequent flux measurements since relative differences can be compared between treatments where ozone and NO reactions will be the same for all treatments. However absolute fluxes of NO may be slightly underestimated on days when ozone concentrations are high, due to the nature of the fieldwork it was not possible to make *in situ* ozone measurements at every sample date.

### 3.2. Microcosm dynamic NO flux system calibration

The NO dynamic microcosm system presented in Chapter 2 required calibration of various pieces of equipment at regular intervals to ensure accurate determinations of nitric oxide flux. The calibration of the two mass flow controllers (MFC) that effectively maintained flow rates through the soil microcosm and dilution zero air was determined at regular intervals.

The mass flow controller used to regulate soil microcosm airflow rate was factory calibrated at 0 to 100 sccm (standard cubic centimeters  $\text{min}^{-1}$ ), a second mass flow controller factory calibrated at 0 to 2.5 slpm (standard litres  $\text{min}^{-1}$ ) was used to regulate and measure the flow rate of the "zero" air dilution.

A Tylan RO-28 (Tylan, USA) flow readout control instrument was modified so that a 0 to 5 volt full-scale deflection indicating the voltage output from the mass flow controllers could be logged using a Campbell 21X data logger.

The offset and multiplier values for the flow rate output determinations were set to zero and one respectively on the Campbell logger program (Campbell Scientific), thus logging a true voltage output from the mass flow controllers.

The voltage output from both mass flow controllers was then logged following changes in RO-28 flow settings. The mass flow controllers were calibrated with the RO-28 using a flow meter (Drycal).

Flow rates through the MFC were measured using a flow meter (Drycal) connected to the outlet side of the mass flow controller, and a pump on the inlet end of the mass flow controller capable of flow rates exceeding  $8 \text{ l min}^{-1}$ .

The Tylan RO-28 flow readout controller was set to a flow rate of zero through the mass flow controller. The flow rate was then measured using the Drycal DC-2 and the mass flow controller voltage readings were logged using the Campbell 21X data logger, the times when flow rates were measured were noted so that logger data could be matched to DC-2 data.

The Drycal DC-2 flow meter was set to take 10 consecutive flow readings and the corrected average of these was recorded; this was repeated 5 times prior to changing any flow setting values on the RO-28, in effect providing five replicates of 10 for flow rate. The RO-28 was set to at least 6 flow set points and average flow measured using the Drycal DC-2 flow meter so that a plot of actual flow rate against mass flow controller voltages could be plotted.

The results for both flow meter calibrations are shown in Figures 23 and 24, representing the 0 to 100 sccm mass flow controller which controlled the flow rate through the soil microcosm and the 0 to 2.5 slpm mass flow controller which controlled the flow rate through the diluting air path.

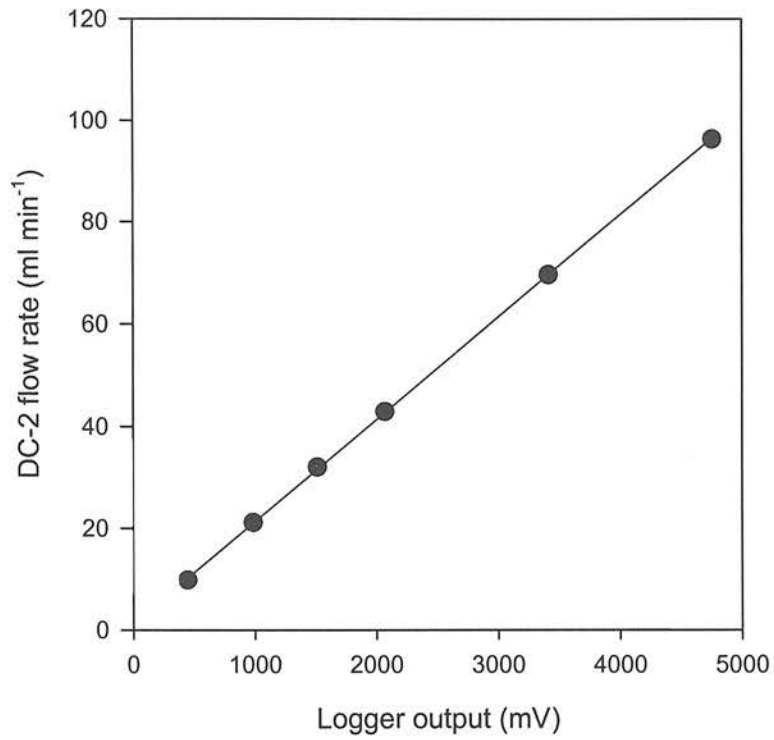


Figure 23. Simple linear regression of data from the 0-100 sccm MFC

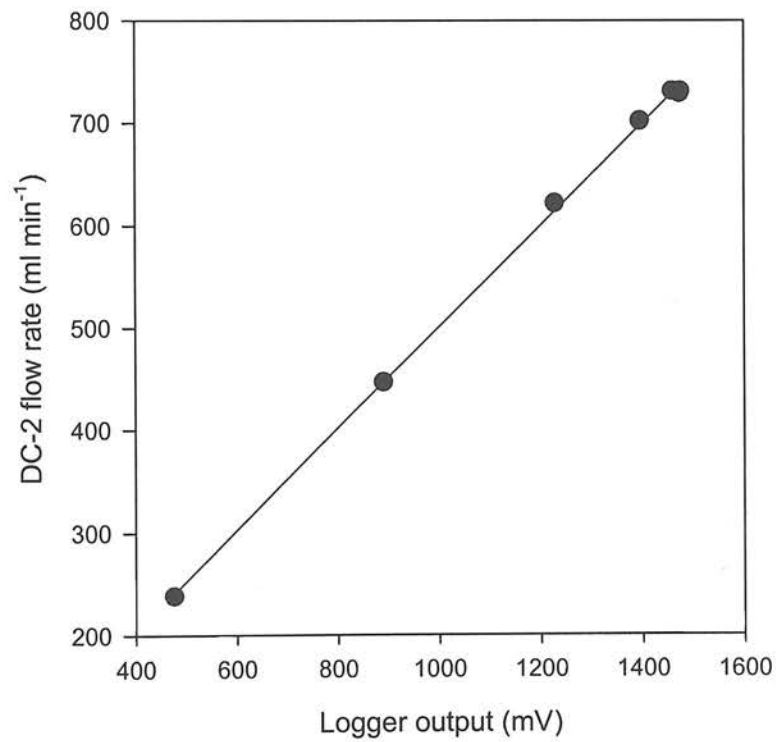


Figure 24. Simple linear regression of data for the 0-2.5 slpm MFC

The regression analysis from the line of best fit in Figures 23 and 24. are shown in Equations 9 and 10. The  $R^2$  values from the linear regressions were 100 and 99.9% respectively ( $p < 0.001$ ).

Equation 9. Regression equation for 0-100 sccm MFC

$$\text{DC-2 flow rate (Y)} = 0.02 (\text{M}) * \text{logger output (X)} + 1.26 (\text{C})$$

Equation 10. Regression equation for 0-2.5 slpm MFC

$$\text{DC-2 flow rate (Y)} = 0.496 (\text{M}) * \text{logger output (X)} + 4.98 (\text{C})$$

The results for the equation of the straight lines following linear regression were then used to provide a multiplier and offset value in the Campbell data logger program so that voltage output could be converted to a correct flow rate in  $\text{ml min}^{-1}$  for each of the mass flow controllers.

### 3.3. Nitrite detection and extraction from soil

To quantify functional diversity it was important to elucidate the two separate steps of nitrification in terms of inorganic N cycling from ammonium through to nitrite and ultimately to nitrate. The soil nitrite pool tends to be highly transient with ammonium oxidation being the rate limiting step for nitrite oxidation, thus soil nitrite concentrations tend to be low.

It was hypothesized that nitrite produced by nitrification of ammonium is very transient in soils and either doesn't accumulate and therefore is not detectable or that abiological reactions with nitrite reduce the concentrations in soil. Two experiments were set up to evaluate the extraction methods used for analysis of inorganic nitrogen from soil and in particular nitrite. One involved extraction of

nitrite following addition of  $\text{NaNO}_2$  at increasing concentrations and another addition of  $\text{NH}_4\text{NO}_3$  followed by nitrite extraction and determination.

Small plastic bags were used to mix the soil (50 g wet weight) and  $\text{NaNO}_2$ , soil inorganic N was extracted using 20 grams wet weight of soil and 100ml of 1M KCl as detailed in Chapter 2.

Soil inorganic N samples were extracted immediately after addition of nitrite at three concentrations of N; low, medium and high concentrations corresponding to  $\text{NO}_2^-$  - N concentrations of 0.05 ppm /  $0.7 \mu\text{g l}^{-1}$ ; 0.5 ppm /  $7 \mu\text{g l}^{-1}$ ; and 1ppm /  $14 \mu\text{g l}^{-1}$ .

The soils were analysed for nitrite following 1M KCl extraction using the Kontron analyser described in Chapter 2 without the reducing agent used in nitrate determinations, this was replaced by 1M KCl.

The results indicated that nitrite was not detectable following extraction from soils using this equipment at any of the nitrite concentrations, it was therefore decided that nitrite analyses should not be carried out on any soil KCl extracts. The continuous flow system (Chemlab Instruments Ltd) and colorimeter described in chapter 2 was however able to detect the nitrite N concentrations of standards from 0.5 to 2.5 ppm in 1M KCl indicating that nitrite was either being abiologically or biologically processed

### **3.4. Quantification of nitric oxide sources following nitrite addition to sterile soil**

During a long term enrichment experiment in which  $\text{NaNO}_2$  was added as a treatment very high NO and  $\text{NO}_2$  concentrations were detected in excess of 100 ppb ( $>1.7 \mu\text{g g}^{-1} \text{h}^{-1}$ ) following addition to soil microcosms. It was therefore necessary to quantify this source of NO since it can be produced as a result of both biological and abiological reactions.

Replicate soil microcosms were set up using sterile and non-sterile soil. Soil sampled from Sourhope control plots was dried and then autoclaved at  $120^\circ\text{C}$  and 30 psi for two consecutive 20 minute periods. Soil was then re-packed in microcosms



(229 g wet weight) following mixing and addition of  $\text{NaNO}_2$  in de-ionised water at  $14 \mu\text{g l}^{-1}$  to a soil WFPS of 50%. The microcosms were then capped and analysed for NO production using the steady state dynamic system described in Chapter 2.

The results in Figure 25 show that following nitrite addition to sterile columns there was a significant increase in NO flux ( $p < 0.05$ ), this was in excess of  $7 \mu\text{g g}^{-1} \text{ dry wt soil h}^{-1}$ . In this case the NO produced was due to abiological chemical reactions. There are problems with using autoclaved soil since many organic compounds are released as well as lysis of bacterial cells containing the necessary enzymes for nitrite oxidation and thus NO production.

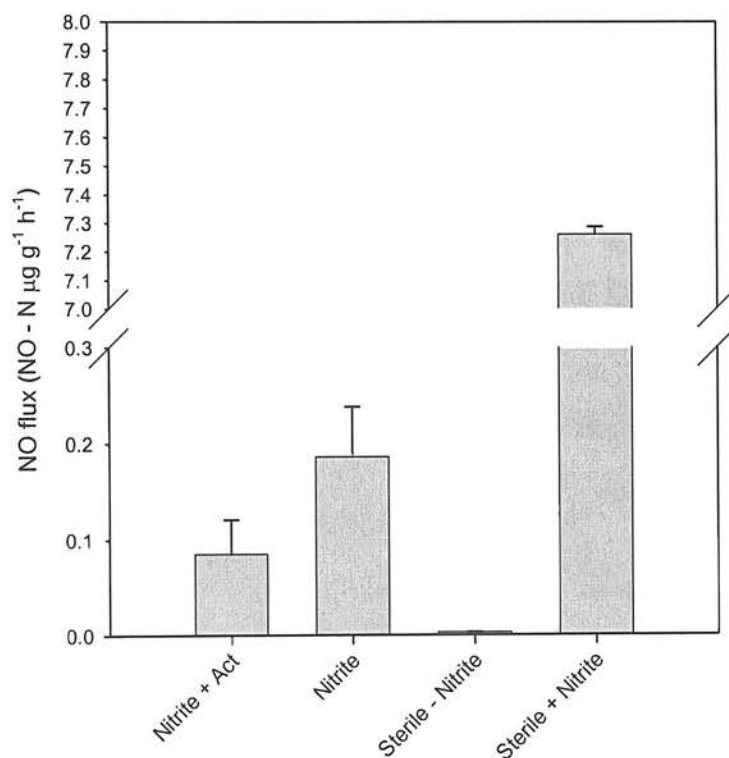


Figure 25. Nitric oxide production following nitrite addition to soil microcosms at 40% WFPS in sterile and non sterile soil microcosms (Act = acetate, nitrite =  $\text{NaNO}_2$ ).

Under non-sterile conditions in columns from the long term enrichment experiment described in Chapter 6, the NO production was at least 10 times that of columns incubated with just ammonium nitrate and therefore whilst some of this production could be biological, it is likely that the majority of production is via abiological reactions.

### 3.5. Effects of soil weight on NO and N<sub>2</sub>O flux in soil microcosms

In order to test the effects of soil weight on NO flux, soil microcosms were set up at 40% WFPS following addition of NH<sub>4</sub>NO<sub>3</sub> at 126 Kg ha<sup>-1</sup> yr<sup>-1</sup> with four soil dry weights (50, 100, 150 and 200 grams). Each soil microcosm was analysed for NO and NO<sub>2</sub> production using the system described in Chapter 2.

The results in Figure 26 show the effects of soil weight on NO flux where the flux is expressed as  $\mu\text{g g}^{-1}$  dry wt soil h<sup>-1</sup> and  $\mu\text{g m}^{-2}$  h<sup>-1</sup>. There was a decrease in flux expressed on both a soil weight and surface area basis with increasing soil weight. The mixing ratio (ppb) of nitric oxide also decreased with increasing soil weight.

The data were not normally distributed, therefore NO flux was log transformed so that simple linear regression could be performed, the results of which are presented in Figure 27.

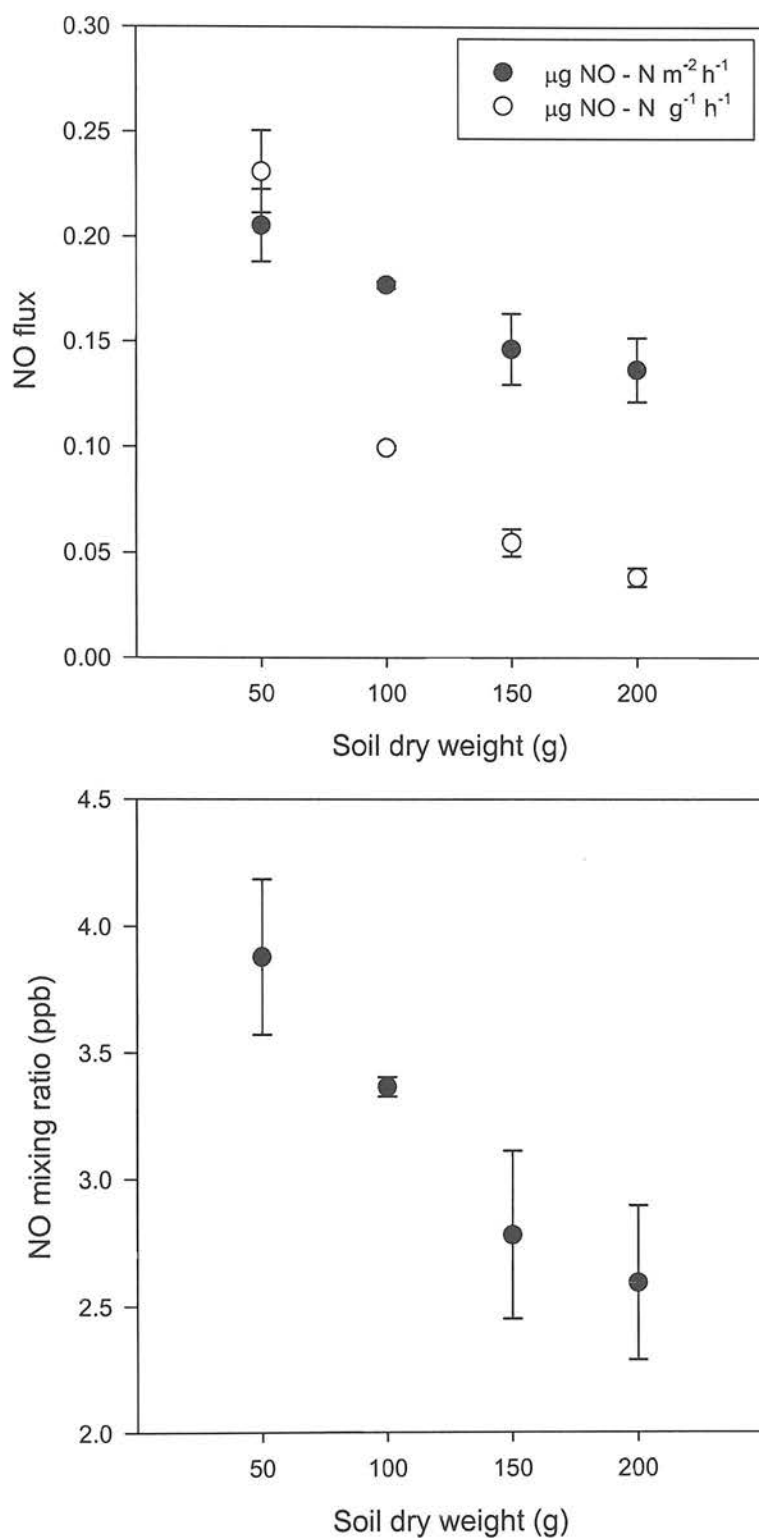


Figure 26. The effects of soil weight on nitric oxide flux calculations (a) and mixing ratios (b) ( $n=2$  for each soil weight)

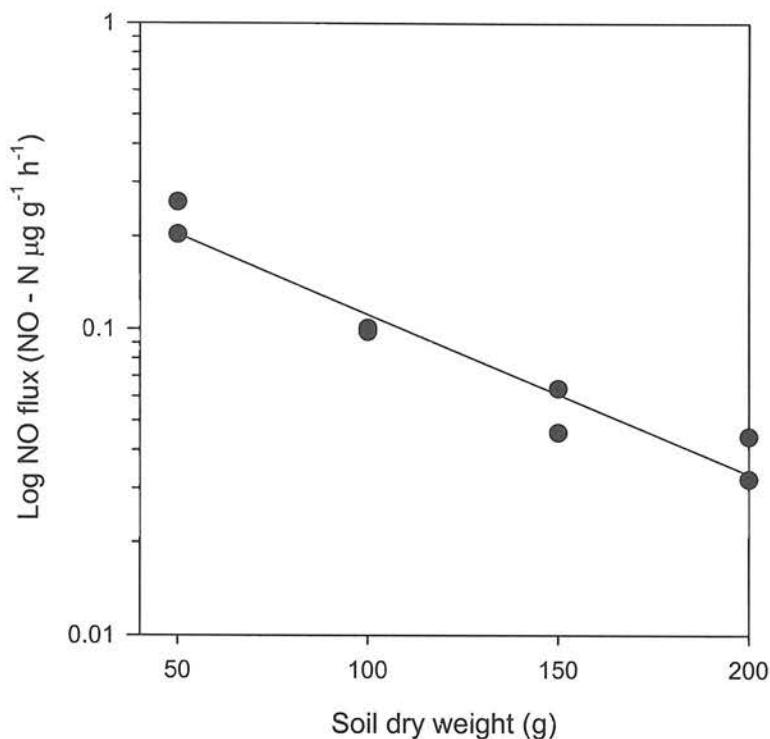


Figure 27. Linear relationship between log transformed nitric oxide flux and soil dry weight ( $n=2$  for each soil dry weight)

The linear regression for the log transformed NO flux gave an  $R^2$  of 93.5% and a  $p$  value of  $<0.0001$ , following one way analysis of variance (Minitab, USA) there was a significant effect of soil weight on nitric oxide flux ( $p = 0.003$ ).

It was therefore necessary to present all microcosm results on a soil weight basis since soil weight was found to have a significant effect on both the mixing ratio and flux of NO, this could be mediated by a number of factors either gas transport mechanisms and or effects of soil depth on production and consumption of nitric oxide.

### 3.6. The effects of sweep air flow rate on NO flux in soil microcosms

The quantification of flux from headspace changes in gaseous concentrations can either be steady or non-steady state, therefore the various parameters governing

flux from these changes need to be optimized so that gas measurements are made during steady state conditions.

With the dynamic steady state system used to calculate NO flux it was necessary to ensure that measurements were made at a sweep air flow rate optimized for steady state gas transfer between soil and sweep air.

Soil sampled from Sourhope control plots was air dried and re-packed (229g dry weight) into soil microcosms (three replicates),  $\text{NH}_4\text{NO}_3$  was added at  $126 \text{ kg ha}^{-1} \text{ yr}^{-1}$  in solution to give a final soil WFPS of 40%.

The sweep air flow rate was then set to flow rates from 0 to  $100 \text{ ml min}^{-1}$  at  $10 \text{ ml min}^{-1}$  intervals and the NO and  $\text{NO}_2$  concentration was determined for each soil microcosm at each flow rate.

The results in Figure 28 show the effects of sweep air flow rate on mean headspace NO concentration changes and the effects of sweep air flow rate on the flux of NO.

There was a significant correlation between sweep air flow rate and headspace concentration of NO after reaching equilibrium ( $r^2 = -0.99$ ,  $p = <0.0001$ ), when looking at the relationship between NO flux expressed as  $\mu\text{g m}^{-2} \text{ h}^{-1}$  and sweep air flow rate there was also a significant correlation ( $r^2 = 0.96$ ,  $p = <0.0001$ ) based on a simple exponential 2 parameter fit.



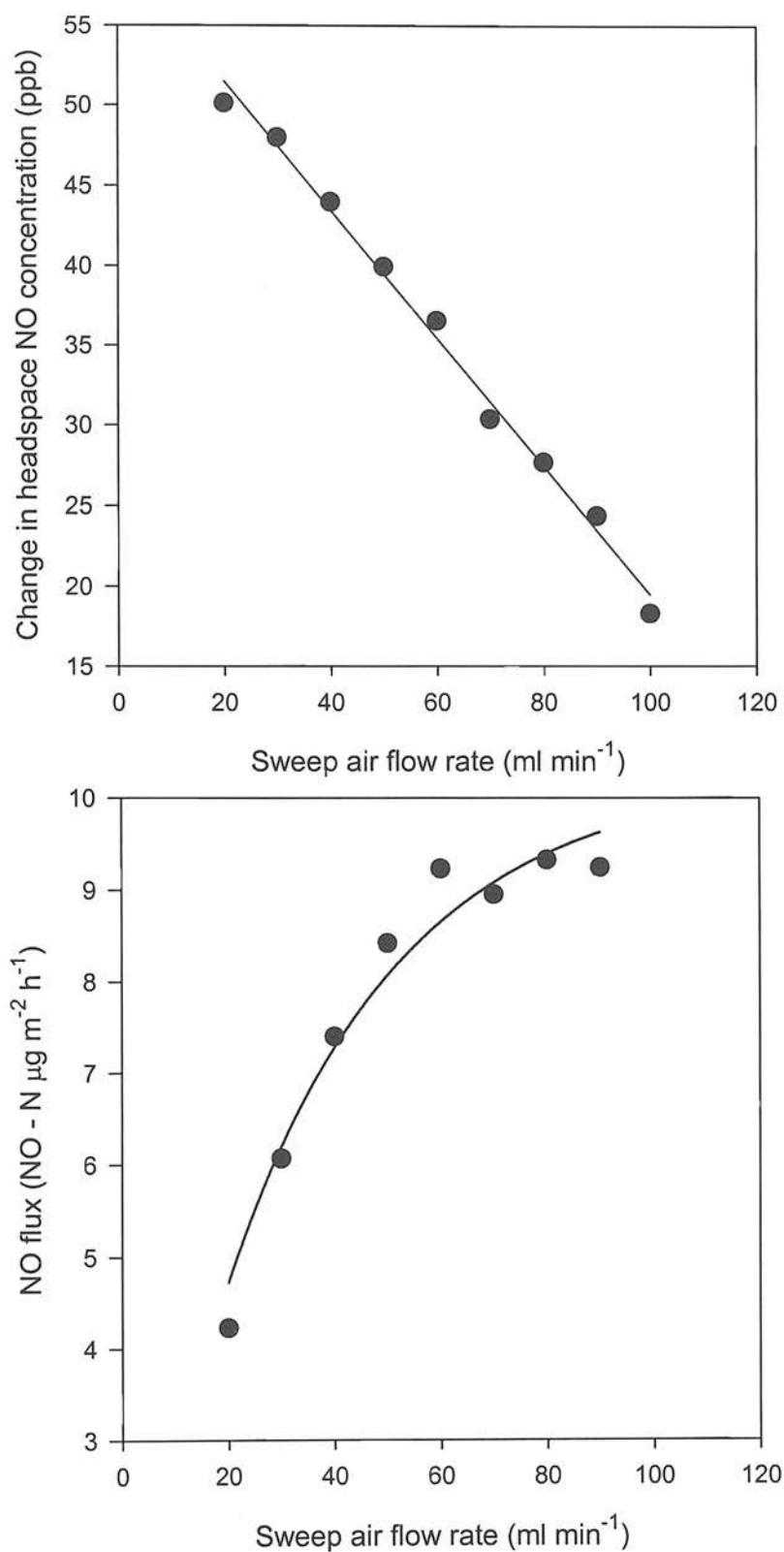


Figure 28. The effects of sweep air flow rate on NO flux in  $\mu\text{g g}^{-1} \text{h}^{-1}$  and NO mixing ratios

When the flux was expressed as  $\mu\text{g g}^{-1} \text{h}^{-1}$  there was a significant negative correlation ( $r^2 = 0.99$ ,  $p = <0.0001$ ) with sweep air flow rate (Figure 27), this again indicates the importance of soil weight on flux of nitric oxide when using a dynamic microcosm system.

Simple linear regression analysis showed that over the flow rates used for these experiments steady state conditions were achieved below  $60 \text{ ml min}^{-1}$  therefore a flow rate of  $40 \text{ ml min}^{-1}$  was selected for all microcosm experiments which enabled a relatively short sample time and allowed response times for the  $\text{NO}_x$  analyzer to be taken into account.

## Chapter Four. Effects of Soil temperature and moisture content on nitric and nitrous oxide emission in soil microcosms

### 4.1. Introduction

With increased global climate change temperature could increase by upto 3-5°C above ambient in Europe (Hantschel *et al.* 1995), this would not only increase rates of biogeochemical cycling but also affect the soil moisture and gas diffusion.

The environmental factors affecting nitric and nitrous oxide emissions are of particular importance to the nitrogen cycle in upland grassland systems, which are strongly influenced by biological activity (Swift *et al.* 1998). Optimum land management regimes and time of fertiliser application need to be carefully planned to prevent excessive loss of N via nitric and nitrous oxide and leaching via nitrate to groundwaters. The source flux for cultivated and un-cultivated grassland estimates for nitric and nitrous oxide are 2.8 and 0.6 to 2.2 Tg N year<sup>-1</sup> respectively, accounting for almost 13.5 and 7.6 to 27.8 % of the total global sources respectively (Davidson, 1991).

Both the processes of nitrification and denitrification can lead to nitric and nitrous oxide production under very different environmental conditions. Two of the major controlling factors over both are temperature and soil moisture content. (Young *et al.*, 2000; Davidson, 1991). These two controlling factors are able to influence microbial processes on several levels and over small to large scales, from the microbial scale and effects on the availability of substrates for growth (Stark and Firestone, 1995) to the landscape scale and diffusion of gases both into and out of the soil (Abassi and Adams, 2000).

Enzymes within both nitrification and denitrification pathways are either expressed or inhibited as a result of several environmental factors (Zumft 1997 and Prosser, 1989); two important environmental factors which regulate enzyme expression and activity and therefore nitric and nitrous oxide production are oxygen availability and temperature (Davidson and Schimel, 1995).

Under anaerobic conditions and therefore high soil moisture content denitrification reduces nitrogen in the form of nitrate to  $N_2$  gas via a series of respiratory enzymes. The first step of denitrification involves nitrate reduction by nitrate reductase, three different forms of nitrate reductase have been isolated from denitrifiers and many bacteria have more than one of these types (Zumft, 1997). The three types of nitrate reductase consist of a periplasmic nitrate reductase in which nitrate is reduced to nitrite outside the cell (NAP); a periplasmic membrane bound nitrate reductase in which the active site is within the cytoplasm (NAR) and therefore nitrate must first be transported into the cell before it can be reduced to nitrite. The two membrane bound nitrate reductases are respiratory enzymes where reduced forms of N are used as terminal electron acceptors. The third nitrate reductase is the soluble cytoplasmic assimilatory nitrate reductase (NAS), one or more of these nitrate reductases may be found within one species of denitrifier and in fact all three have been isolated from *Paracoccus denitrificans* (Zumft, 1997).

Regulation of the three different nitrate reductases may be mediated by low oxygen concentrations and presence of a respirable N oxide, in particular NAP is expressed under aerobic conditions whereas NAR is expressed under anaerobic conditions (Berks *et al.*, 1995 and Zumft, 1997).

#### 4.1.1. Effects of soil moisture content

The effects of soil moisture on soil microbial processes are mediated via enzyme expression in both the nitrification and denitrification pathways, which are strictly dependant upon oxygen. Under anaerobic conditions and high soil moisture content full reduction of nitrate through to di-nitrogen gas occurs via denitrification. Not only does the concentration of oxygen result in expression or non expression of an enzyme but also the type of enzyme as described previously in this chapter. The model for nitric and nitrous oxide emissions via nitrification and denitrification proposed by Davidson (1991) is show in Figure 29.

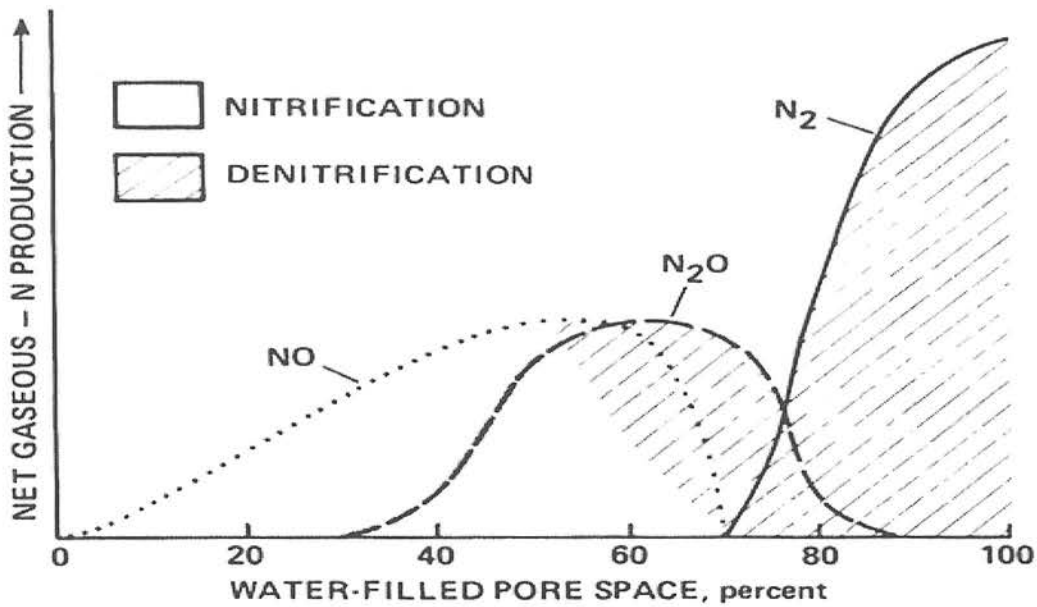


Figure 29. Model of nitric and nitrous oxide production as a function of WFPS from Davidson (1991)

#### 4.1.2. Effects of soil temperature

The studies on the effects of temperature on biogeochemical cycles have been well studied, but perhaps not at temperatures similar to those in upland Scottish soils. Sourhope has a yearly mean temperature below 15°C. The optimum temperature for nitrification rates of soils incubated at different temperatures tend to correlate with the temperature of their origin (Dalias *et al.* 2001) and inversely with soil moisture contents (Peterjohn *et al.*, 1994).

Cookson *et al.*, (2002) found that gross nitrification rates in unamended grassland soils were actually inhibited during initial incubation following a temperature change of more than 5°C.

Soil environmental conditions in the form of moisture content and temperature will affect the partitioning of N transformation processes via either nitrification or denitrification under aerobic and anaerobic conditions respectively



and the rates at which these processes occur. The objectives of these studies were to control both soil moisture content and temperature over a range of conditions to favour both nitrification and denitrification and then quantify emissions of nitric and nitrous oxide as a result of both processes to provide data to help rationalise field fluxes of nitric and nitrous oxide and as a test of the WFPS model of Davidson (1991).

## **4.2. Materials and Methods**

### **4.2.1. Soil sampling and field site**

Soil monoliths (30 (l) x 20 (w) x 10 (d) cm) were sampled from five control treatment destructive sampling sub-plots at the Sourhope experimental site on 13th June 2000. The litter layer was removed from the monoliths prior to drying. The soil was sieved and dried to a re-packed soil moisture content below 30% WFPS (229g dry weight re-packed as in chapter 2) using a large drying oven at 20°C. Soils were then stored at 5°C until required.

### **4.2.2. Soil moisture experiment**

Pre-dried soil was acclimatized at 15°C prior to re-wetting and re-packing to minimize the effects of changing temperature from storage at 5 to that of the experimental temperature of 15°C, so that moisture rather than temperature effects were tested. Soil was weighed into aluminum trays (229 g dry weight equivalent) prior to addition of de-ionised water.

De-ionised water was added to dry soil volumetrically, after first calculating the difference between the volumetric moisture content of the intended re-packed soil WFPS of 30, 40, 50, 60, 70 and 80% and initial soil moisture content following soil drying. The de-ionised water was applied as a fine spray containing 0.82 ml of a 1M solution of  $\text{NH}_4\text{NO}_3$  whilst the soil was continuously mixed, the N was applied as a

spray throughout the soil prior to re-packing rather than spraying on the surface after packing to prevent the development of large aerobic and anaerobic macrosites.

Triplicate soil microcosms were re-packed at each soil WFPS. Throughout the experiment microcosm lids were left half covering the top of the column to prevent excess moisture loss and allow gas diffusion into and out of soil core. All columns were incubated in a constant temperature room at 15°C. Each re-packed soil microcosm was weighed at the start of the incubation period and their soil moisture contents maintained gravimetrically with de-ionised water.

Destructive samples were re-packed in 125ml specimen cups at the same moisture contents as the soil columns with equivalent N addition rates on a per g dry weight soil basis. The moisture content of these was also maintained gravimetrically.

Fluxes of nitric and nitrous oxide flux were determined following N addition on the following days 1, 2,3,6,9 and 27. The nitrate and ammonium concentrations of destructive samples were determined following KCl extraction using methods described in Chapter 2.

#### **4.2.3. Soil temperature experiment**

Pre-dried sieved soil was divided into 300 g bags and stored at 6 temperatures (1, 5, 10, 15, 20 and 25°C) for 2 days prior to re-packing and re-wetting at 80 or 30% WFPS to quantify effects of temperature on denitrification and nitrification respectively under anaerobic and aerobic conditions. Conviron units were used for 10, 20 and 25°C in which air moisture contents were maintained at atmospheric, a freezer incubator was used for 1°C incubations (Equipment) and a fridge freezer and constant temperature room for the 5 and 15°C incubations respectively.

Following two days of acclimatization soil microcosms were repacked as described in Chapter 2 and then immediately returned to the incubation temperature.

All gaseous measurements were carried out in the constant temperature room; soil microcosms were insulated using lagging and a box containing foam insulation to prevent increases or decreases in temperature during gas measurements.

#### 4.2.4. Gaseous N determination and inorganic N analyses

The nitric and nitrous oxide production was determined after 1, 2, 5, 7 and 14 days following initial N addition and re-packing. Nitric oxide measurements were made prior to nitrous oxide static chamber measurements. Nitrous oxide was measured using ECD gas chromatography and nitric oxide using chemiluminescence as described in Chapter 2.

### 4.3. Results

The results show that soil temperature and moisture contents have an effect on both nitric and nitrous oxide production, however the effects of temperature are very different at the optimal soil moisture contents for nitrification and denitrification respectively.

#### 4.3.1. Effects of soil moisture content

The trace gas fluxes of nitric and nitrous oxide are shown in Figure 30. The nitrous oxide emission from soil microcosms did not significantly increase with increasing soil moisture content apart from on day one (ANOVA,  $p < 0.0001$ ) following addition of  $\text{NH}_4\text{NO}_3$  (N). The maximum production of nitrous oxide was  $9.942 \times 10^{-3} \mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{h}^{-1}$  at 80% WFPS (Figure 30(a)). This then decreased to  $2.09 \times 10^{-6} \mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{h}^{-1}$  after 27 days of incubation. There were no significant decreases in the flux of nitrous oxide with time at any soil moisture content (ANOVA,  $p = 0.4$ ). At soil moisture contents below 80% WFPS the nitrous oxide

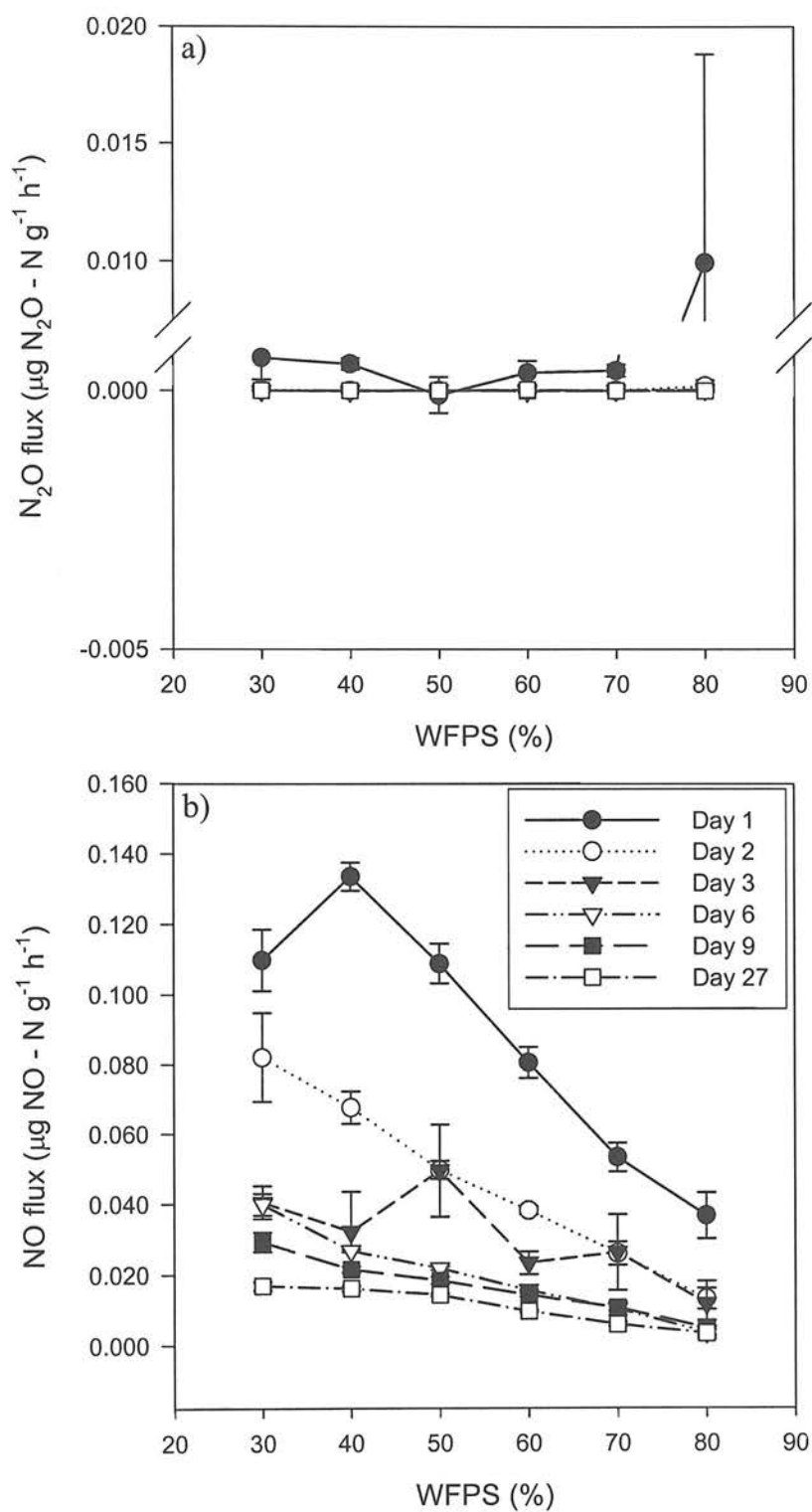


Figure 30. Effects of soil moisture content on the nitrous (a) and nitric oxide (b) flux in soil microcosms incubated at 15°C following addition of  $NH_4NO_3$  (error bars  $\pm$  S.E.)

flux averaged between  $2.395$  and  $9.714 \times 10^{-7} \mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{h}^{-1}$  over all days at 50 and 60% WFPS respectively.

The flux of nitric oxide was greatest at 40% WFPS with a flux on day one following N addition of  $0.134 \mu\text{g NO} - \text{N g}^{-1} \text{h}^{-1}$  (Figure 30 (b)). There was a significant effect of soil moisture content on nitric oxide flux (univariate ANOVA,  $p < 0.0001$ ), with the lowest flux at 80% WFPS (Figure 30 (b)). This decreased throughout the experiment with time, with a significant decrease in nitric oxide flux at all soil moisture contents (univariate ANOVA,  $p < 0.0001$ ).

Nitric oxide production also showed a significant negative correlation with soil WFPS, the data from day 2 of the incubation are show in Figure 31.

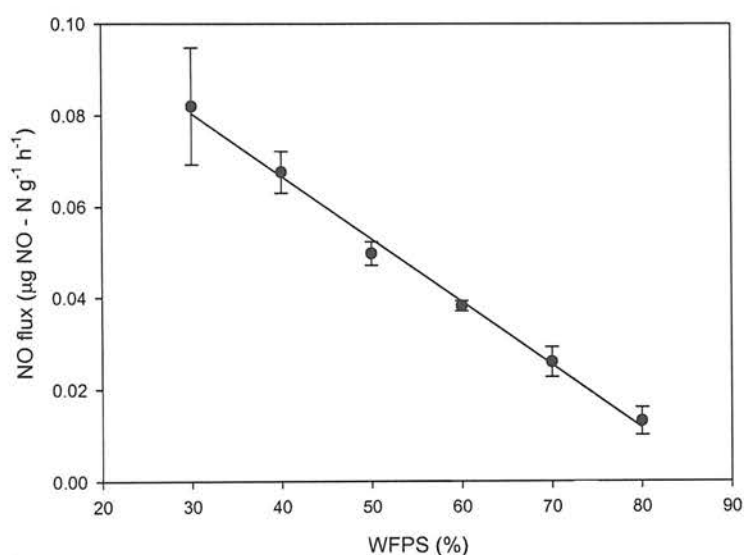


Figure 31. Simple linear regression between soil moisture content and nitric oxide flux in soil microcosms incubated at  $15^\circ\text{C}$  2 days post  $\text{NH}_4\text{NO}_3$  addition ( $r^2 = -0.88$ ,  $p < 0.001$ )

Soil exchangeable ammonium and nitrate concentrations either decreased or increased depending upon the soil moisture content (Figure 32).



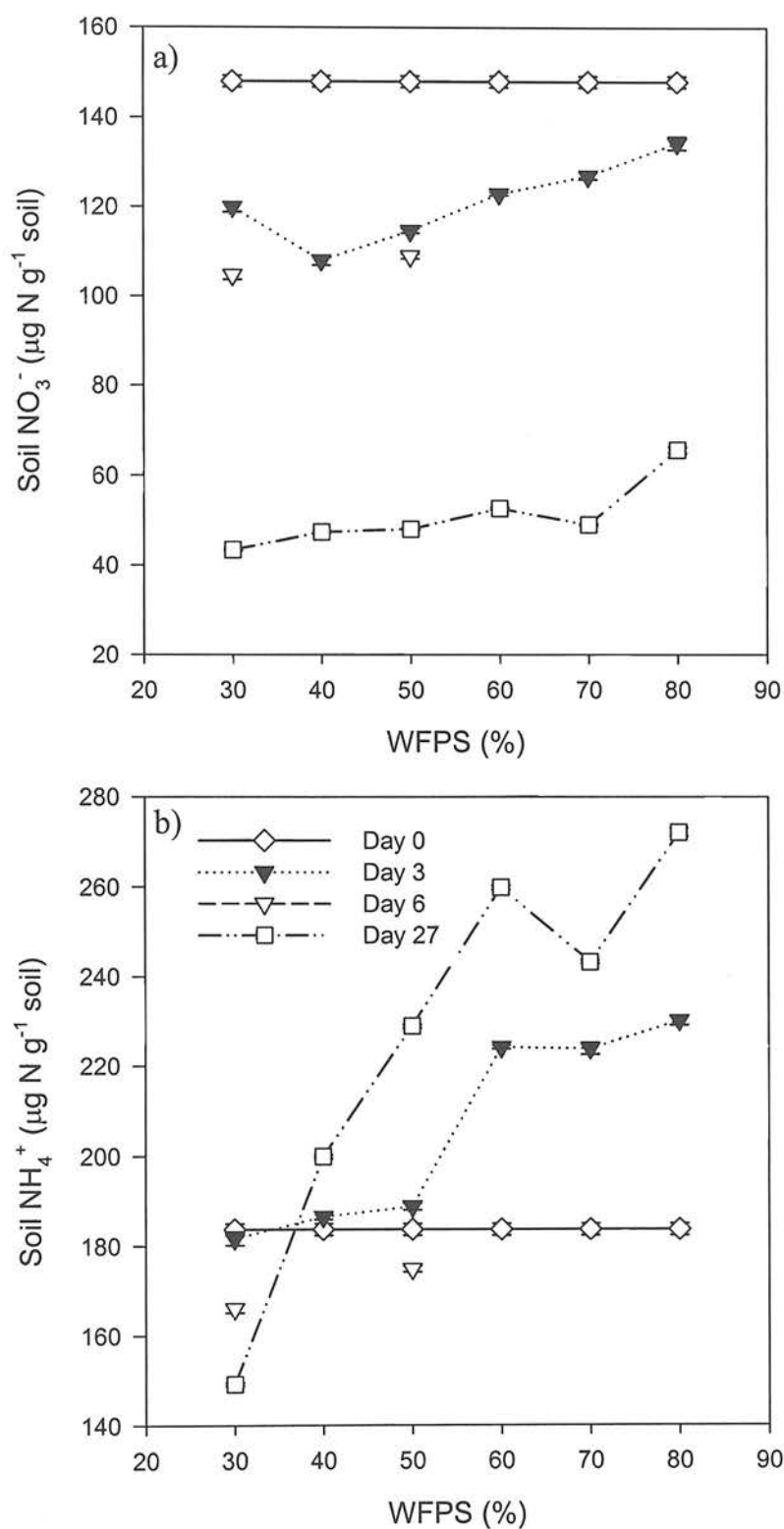


Figure 32. Effects of soil moisture content on soil nitrate (a) and ammonium (b) concentrations in soil microcosms incubated at 15°C following addition of  $\text{NH}_4\text{NO}_3$

Nitrate concentrations decreased significantly (GLM,  $p = < 0.0001$ ) after addition of N at all soil moisture contents, although there were no significant effects of soil WFPS on soil nitrate concentrations on any one day (GLM,  $p = 0.162$ ).

At 30% WFPS there was a significant (GLM,  $p = < 0.038$ ) decrease in the ammonium pool following N addition (Figure 32); which corresponded to high nitric oxide flux shown in Figure 33-c.

The soil ammonium pool significantly increased at all other soil moisture contents throughout the course of the 27 days of incubation. Despite increasing soil ammonium concentrations at 40 to 80% WFPS, the nitrate concentrations decreased from the initial concentration of  $147.92 \mu\text{g} (\pm \text{S.E. } 1.26) \text{ NO}_3^- - \text{N g}^{-1}$  dry weight of soil. The nitrate reduction was greatest in microcosms incubated at soil moisture contents of 30 and 40% WFPS (Figure 32).

Figures 33 a-d show the effects of soil moisture content and incubation time on the correlation between soil exchangeable nitrate concentrations and nitric and nitrous oxide flux.

Generally with increasing incubation time, nitrate concentrations decreased. On day 27 there was a negative correlation between soil nitrate and nitric oxide flux (Figure 33-a) which also corresponded to increasing soil moisture content.

The soil nitric and nitrous oxide flux showed different responses to changing soil nitrate concentrations (Figure 33 a-d); where the nitrous oxide flux increased with increasing soil nitrate (Figure 33-b and d); correlating with soil moisture contents ( $r^2 = 0.983$ ,  $p = 0.003$ ) (Figure 33-d).

The soil ammonium pool increased with time and did not correlate with either nitric or nitrous oxide flux (Figures 34 a-d). Nitrous oxide fluxes were highest at higher soil moisture contents with relatively high soil ammonium concentrations.

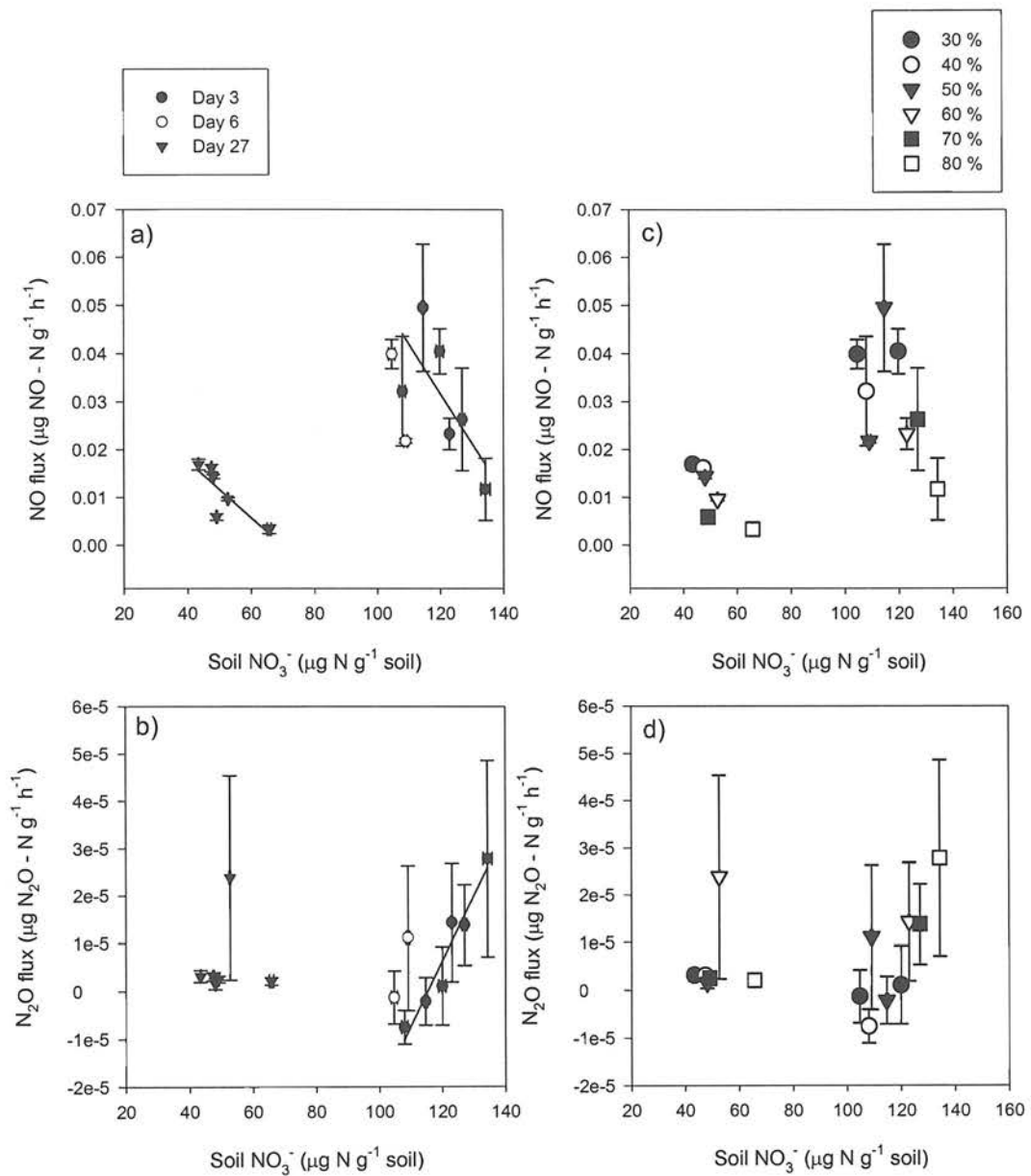


Figure 33. Average nitric and nitrous oxide fluxes and soil exchangeable nitrate concentrations as a function of incubation time a) and b) and soil moisture content c) and d) following  $\text{NH}_4\text{NO}_3$  addition to soil microcosms incubated at  $15^\circ\text{C}$ .

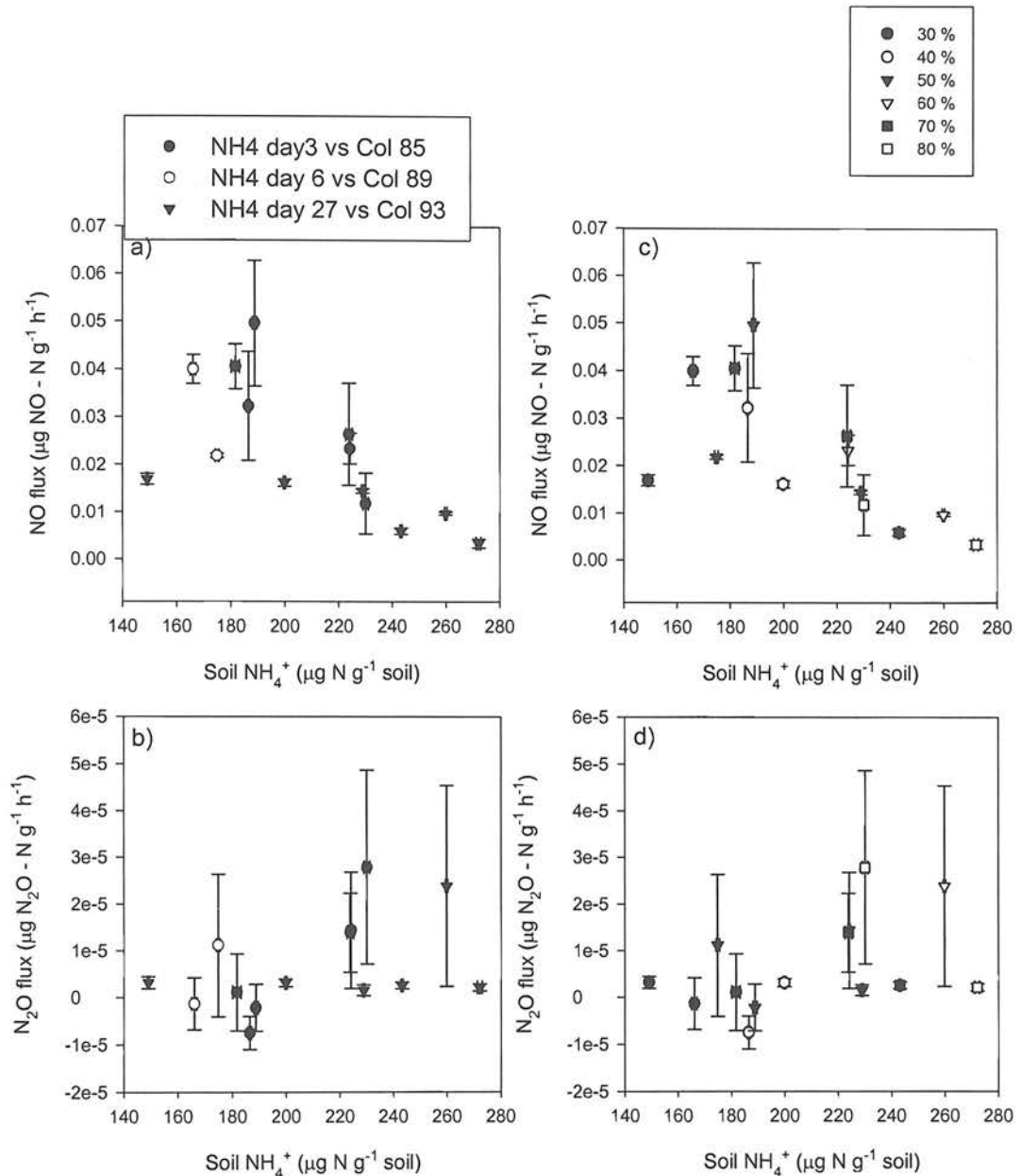


Figure 34. Average nitric and nitrous oxide fluxes and soil exchangeable ammonium concentrations as a function of incubation time a) and b) and soil moisture content c) and d) following  $\text{NH}_4\text{NO}_3$  addition to soil microcosms incubated at  $15^\circ\text{C}$ .

### 4.3.2. Effects of soil temperature

The effects of soil temperature were very different at two contrasting soil moisture regimes. The flux of nitrous oxide was comparatively low at both 40 and 80% WFPS however nitrous oxide did show a temperature optimum at 1 to 15 and 1 to 10°C at 40 and 80 % WFPS respectively (Figures 35 a and c).

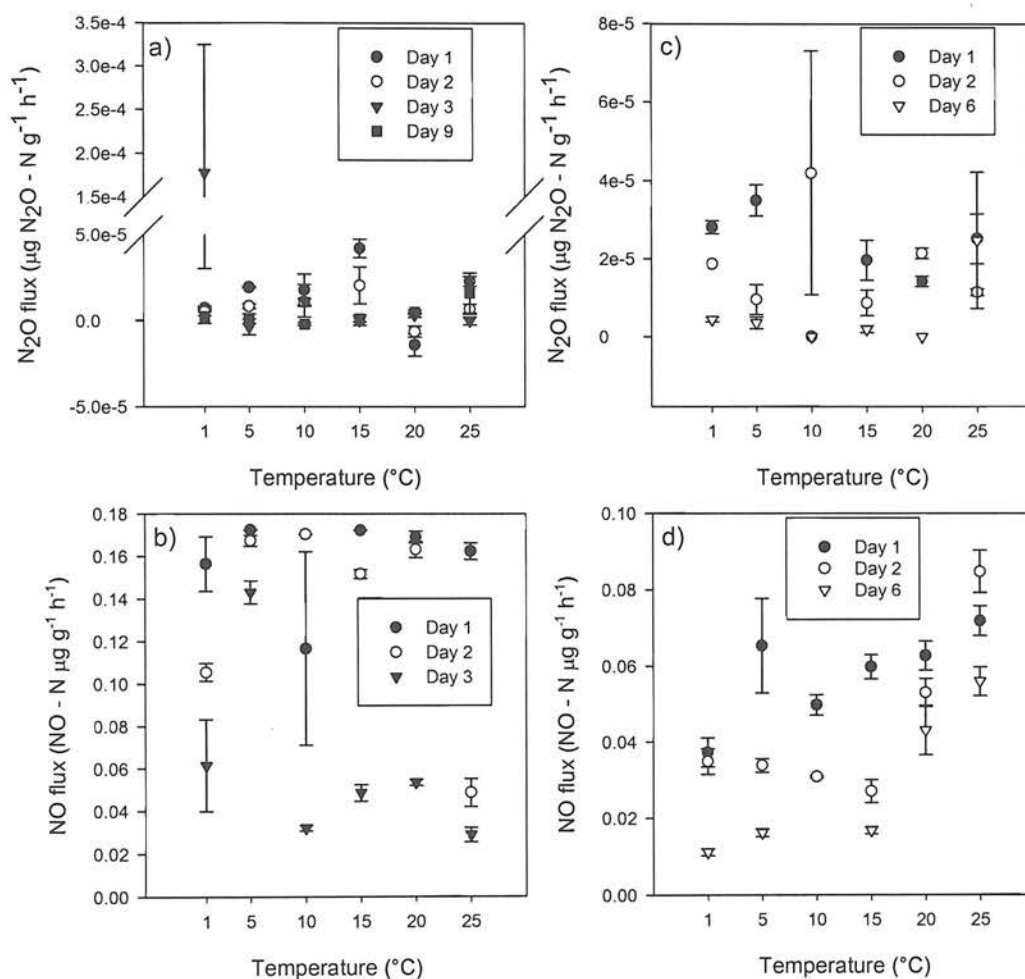


Figure 35. Effects of soil temperature on nitric and nitrous oxide flux at 40 (a) and (b) and 80% (c) and (d) WFPS in soil microcosms following addition of  $\text{NH}_4\text{NO}_3$ .



The flux of nitric oxide was significantly greater at 40 % WFPS than at 80% WFPS (GLM,  $p = <0.001$ ) for all temperatures after one day of temperature incubation (Figures 35 b and d).

The increased nitric oxide flux auto-correlates with increased nitric oxide headspace mixing ratios, where one is determined from an increase in the other; however, Figure 36 a and b shows that the effects of soil temperature and incubation time on nitric oxide flux at different soil moisture contents is significantly different.

At the low soil moisture content the nitric oxide flux was greatest at lower soil temperatures on day 1 and day 2 following addition of ammonium nitrate, with the lowest flux at 25°C.

The opposite was true for the flux of nitric oxide in microcosms incubated at 80% WFPS where increasing temperature resulted in increased production and flux of nitric oxide (Figure 36 b). With time the ratio of nitric oxide flux to nitric oxide mixing ratio also changed at 40% WFPS with lower nitric oxide flux similar to those at 80% WFPS after 2 days of incubation.

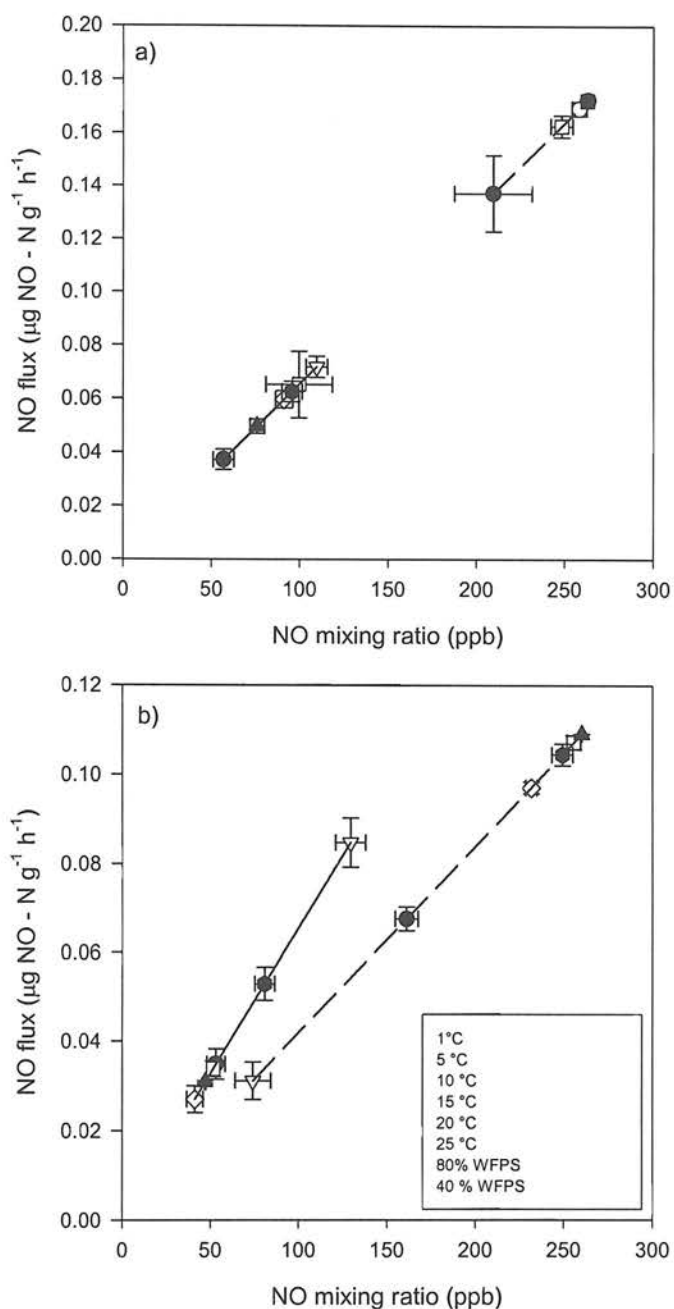


Figure 36. Effects of soil temperature on soil microcosm NO flux and NO mixing ratios at 40 and 80% WFPS on day 1 (a) and day 2 (b) following addition of  $\text{NH}_4\text{NO}_3$ . (error bars = SE,  $n = 3$  for both x and y data)

The apparent  $Q_{10}$  values for both nitric and nitrous oxide were determined (emission rate at  $T^{\circ}\text{C}$ /emission rate  $T - 10^{\circ}\text{C}$ ) at both 40 and 80 % WFPS over a range of temperature intervals (Table 3). At lower temperature ranges and lower soil WFPS the effects of temperature resulted in a greater increase in nitrous oxide emissions over the 1 – 10 and 5 – 15°C ranges with  $Q_{10}$  values of 2.43 and 2.16 respectively; however nitric oxide  $Q_{10}$  values were greater over the 10 – 20°C range (Table 3).

With increased soil moisture content the  $Q_{10}$  values for nitrous oxide were greater over the 15 – 25°C range with no effect of temperature on the nitric oxide  $Q_{10}$  values which averaged 1.18 ( $\pm\text{S.E.} = 0.08$ ).

*Table 3. Apparent  $Q_{10}$  values for nitric and nitrous oxide emissions in soil microcosms measured on day one following addition of  $\text{NH}_4\text{NO}_3$  at 40 and 80% WFPS*

Temperature interval °C	$Q_{10}$ at 40% WFPS		$Q_{10}$ at 80% WFPS	
	NO	$\text{N}_2\text{O}$	NO	$\text{N}_2\text{O}$
1 – 10	0.75	2.43	1.33	0
5 – 15	1	2.16	0.91	0.56
10 – 20	1.45	-0.8	1.26	-
15 – 25	0.94	0.54	1.20	1.28

## 4.4. Discussion

### 4.4.1. Soil moisture

The effects of soil moisture content on the nitric and nitrous oxide emission from soil have been well studied (Fierer and Schimel, 2002; Stark and Firestone, 1995; Davidson 1991; Low *et al.*, 1997; G  dde and Conrad, 1998; Smith *et al.*, 2003), it is generally accepted that as soil moisture contents increase then nitrous oxide production increases as a result of denitrification until nitrous oxide is further reduced to  $N_2$  under completely anaerobic conditions (Zumft, 1997). Conversely at low soil moisture contents denitrification is inhibited by oxygen and nitric and nitrous oxide are produced via nitrification (Zumft, 1997; Prosser 1989).

Results from the WFPS incubations are in agreement with those from other studies (Webster and Hopkins, 1996) and indicate that increasing soil moisture contents from 30 to 80% WFPS significantly decrease emissions of nitric oxide and increase emissions of nitrous oxide but only at 80% WFPS.

The nitric oxide data would fit with the model proposed by Davidson (1991) in which nitric oxide is the dominant source gaseous N emitted from soils below 60% WFPS as shown in Figure 37.. However the measured emission of nitrous oxide was only significant at 80% WFPS whereas the greatest emissions predicted by Davidson (1991) were at 60% WFPS. Field measurements of nitrous oxide following fertilizer additions by Abbasi *et al.* (2000) also indicated that maximum nitrous oxide emissions occur above that predicted by Davidson (1991), the greatest nitrous oxide flux was at 84% WFPS in agreement with these data.

The positive correlation between nitrous oxide flux and soil nitrate concentrations with increasing soil WFPS would indicate that denitrification was the source of nitrous oxide at higher soil WFPS with nitric oxide produced via nitrification (Davidson, 1991).

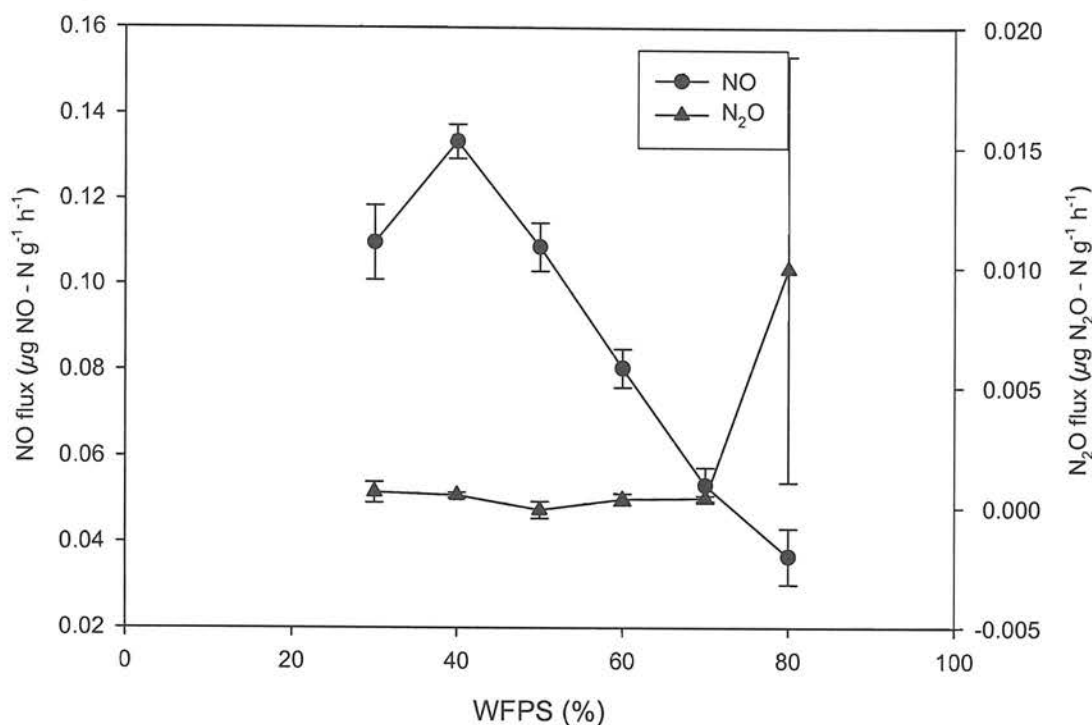


Figure 37. Effects of soil WFPS on nitric and nitrous oxide flux following  $\text{NH}_4\text{NO}_3$  addition to soil microcosms at  $15^\circ\text{C}$

The change from nitric to nitrous oxide flux with increasing soil moisture content did not fit the model proposed by Davidson, however; with re-packed soil microcosms the soil was sieved which may not facilitate formation of anaerobic micro sites, as would be the case in the field with a heterogeneous soil structure. Soil structure plays an important role in the formation of anaerobic micro sites and if this is broken down upon sieving and re-packing then micro sites may not form so readily.

Increasing soil ammonium concentrations with significant decreases in the soil nitrate pool at 80% WFPS; coupled to a corresponding high flux of nitrous oxide but not nitric oxide, suggests that denitrification (predominantly an anaerobic process) is a dominant source of nitrous oxide at high soil moisture contents.



The increase in soil ammonium pool with increased soil WFPS above 30% may be the result of increased mineralization of organic nitrogen following initial re-wetting. Rewetting-drying cycles have been shown to induce significant effects on microbial carbon and nitrogen dynamics; Fierer and Schimel (2002) noted increased nitrification potentials in grassland soils following drying-re-wetting periods, although only after several of these cycles.

Both nitric and nitrous oxide flux decreased following initial N additions at all soil moisture contents, this was coupled to a decrease in the soil ammonium pool with time at 30% WFPS; this would indicate that nitrification is the dominant process at low soil moisture contents where inorganic ammonium is oxidised to nitrate with nitric oxide produced as a by-product of ammonium oxidation to nitrite (Ruscow *et al.*, 2000). Nitrous oxide fluxes were very low at all soil moisture contents below 80% WFPS despite increased reduction of nitrate after 27 days incubation at these moisture contents. These data may be explained by aerobic denitrification as Ruscow *et al.* (2000) presented data for unsaturated soils following a series of stable isotope  $^{15}\text{N}$  additions in which they attributed small fluxes of nitrous oxide to aerobic denitrification according to Poth and Focht (1985).

#### 4.4.2. Soil temperature

The complex interactions of soil moisture and temperature were evident in the data presented for soil temperature incubations. Most biological enzymatic reactions increase with increasing temperature as a result of enzyme kinetics (Morris, 1974) however the soil temperature incubation data indicate temperature optima for nitric and nitrous oxide flux. Different species of bacteria isolated into pure culture from soil have very different temperature optima; and indeed sulphur oxidizers in deep sea thermal vent regions and psychrophilic bacteria are able to grow at extremely hot ( $>110^{\circ}\text{C}$ ) and cold ( $12^{\circ}\text{C}$ ) conditions as a result of specialized adaptive physiological features which have evolved over many millions of years (Atlas and Bartha, 1998).

The optimum temperature for greatest nitric oxide emissions at 40% WFPS were between 5 and 15°C, the mean yearly soil temperature (0-10 cm depth) in the field was 10°C whereas optimum temperature at 80% WFPS was 10°C, the apparent  $Q_{10}$  values for the emission of nitric oxide were also greatest over the 10 – 15°C range at 40% WFPS

Emission of nitric oxide was also greater at 40 than 80% WFPS over all temperatures, these data are similar to the fluxes of nitric oxide in the WFPS incubation experiments. Despite increasing nitric oxide emissions with increasing temperature at 80% WFPS the apparent  $Q_{10}$  values were not significantly different. Increasing nitric oxide flux with temperature at 80% WFPS could therefore be the result of drying effects due to higher soil temperatures, where emission of nitric oxide is mediated by decreasing soil moisture at the soil surface as the column dries out, providing optimal conditions for nitrification with low soil moisture content and high soil ammonium concentrations as a result of initial N additions.

The effects of temperature on nitrous oxide emissions at low soil moisture contents were more pronounced at 15°C as shown in Figure 35 and the apparent  $Q_{10}$  values were also greatest at the lower soil temperature ranges between 1 to 10 ( $Q_{10}$  2.43) and 5 to 15°C ( $Q_{10}$  2.16). These data are similar to those for other grassland soils in which nitrous oxide emissions were greatest at 18°C following fertiliser and water addition to soil cores (Dobbie and Smith, 2001) with  $Q_{10}$  values of 3.7 and 2.3 over 5 – 12 and 12 – 18°C temperature ranges.

Dobbie and Smith (2001) attributed decreasing  $Q_{10}$  values with increasing temperature to enhanced water loss during incubation and indeed above these temperature ranges at 40% WFPS these studies showed similar trends (Table 3).

However, at 80% WFPS  $Q_{10}$  values for nitrous oxide emissions were greatest at the higher temperature range of 15 – 25°C in these studies which are not comparable to those obtained by Dobbie and Smith (2001) at roughly the same soil WFPS (88%) and incubation temperatures. Nitrous oxide emissions at 80% WFPS also showed 2 maxima of 5 and 25°C (Figure 35), this probably reflects true optimum temperature at 5°C with effects of drying at 25°C and decreasing soil

moisture. The effects of soil moisture on gas transport have been reported previously (Davidson and Schimel, 1995) and in an open system it is likely that higher temperatures did result in “artificially” enhanced emissions of nitrous oxide due to increased diffusion out of the soil rather than true temperature effects on microbial processes. Optimum temperatures for nitric and nitrous oxide indicated different maxima at low and high soil WFPS, this may be complicated by the effects of drying but could also indicate two different processes were responsible for emissions at 40 and 80% WFPS.

## Chapter Five. Effects of specific inhibitors on soil microcosm nitric and nitrous oxide flux.

### 5.1. Introduction

Addition of nitrogen and lime to soil has been shown to significantly affect the soil chemistry by resulting in either greater availability of inorganic nitrogen following fertilizer application and/or increasing soil pH from an acidic to an alkaline pH (Kyveryga *et al.*, 2004). The conditions under which autotrophic and heterotrophic processes occur depend greatly upon soil type, redox potential, carbon and nitrogen availability and pH (Kurakov *et al.*, 2001), land management in the form of nitrogen and lime addition may change one or more of these soil characteristics and therefore mediate a change in the dominant microbial processes of the nitrogen cycle and in turn emission of nitric and nitrous oxide. This study aimed to determine the direct effects of land management on the dominant microbial processes responsible for nitric and nitrous oxide flux, the treatments were pre-determined by the Soil Biodiversity programme to reflect common agricultural practices in upland grasslands. Various studies have used specific inhibitors of nitrification to separate autotrophic from heterotrophic production of nitric and nitrous oxide. The specific aims of this study were to use the autotrophic nitrification inhibitor dicyandiamide (DCD) and the heterotrophic inhibitor cyclohexamide coupled with isotope pool dilution to determine the dominant sources of nitric and nitrous oxide in the Sourhope field treatments of control, lime, nitrogen and nitrogen plus lime.

#### 5.1.1. Specific inhibitors of nitrification

The autotrophic inhibitor DCD is predominantly used by farmers to inhibit the rates of nitrification following application of ammonium nitrate based fertilisers in the field. The DCD molecule is water soluble dimeric form of cyanamide and inhibits the ammonium monooxygenase enzyme (AMO) of autotrophic nitrifiers.

The DCD inhibition of AMO is reversible. A few weeks after application, DCD is degraded by bacteria to ammonium and CO<sub>2</sub> following catalysis by iron oxides, this ammonium can then potentially be a substrate for nitrification (Amberger, 1981).

Nitrapyrin, another inhibitor of autotrophic nitrification, is a heterocyclic compound, which inactivates the AMO of autotrophic nitrifiers; although its exact mechanism is unknown (McCarty, 1999). Nitrapyrin is however not applicable as a selective inhibitor in organic rich acid soils due to sorption and therefore DCD was used in these studies.

The use of cyclohexamide in soil microcosm studies was carried out by Castaldi and Smith (1998) where heterotrophic nitrification was determined using cyclohexamide. The cyclohexamide inhibits protein synthesis and thus heterotrophic nitrification.

To enhance the use of specific inhibitors such as acetylene for quantifying sources of nitric and nitrous oxide; <sup>15</sup>N pool dilution has also been used (Barraclough and Puri, 1995 and Davidson *et al.*, 1993), recently studies have used GC/CF IRMS to quantify nitrous oxide production from nitrification and denitrification using <sup>15</sup>N labelled nitrate and/or ammonium (Stevens *et al.*, 1993; Panek *et al.*, 2000; Russow *et al.*, 2000).

Pool dilution methods are often used in conjunction with acetylene blockage and involve the addition of acetylene to a soil headspace at 0.01% v/v to inhibit nitrification. Acetylene inhibits the AMO enzyme of nitrifiers following oxidation to a highly reactive product causing irreversible inhibition (De Boer *et al.*, 2001), however there are difficulties in using acetylene at high soil moisture contents due to the effects of the solubility of gases in solution which may not inhibit bacteria within microsites.

## 5.2. Materials and methods

### 5.2.1 Soils and sampling

Soils were sampled from the Sourhope as 20 by 20 by 10cm monoliths from the destructive treatment plots of control (C), lime (L), nitrogen (N) and nitrogen plus lime (N+L), monoliths were used rather than intact soil cores or *in situ* measurements so as to reduce the effects of soil heterogeneity on nitric and nitrous oxide flux. The soil monoliths were then broken up and sieved, followed by drying to a soil moisture content below 40% WFPS using methods as described in chapter 2. In addition to the four Sourhope treatments an autotrophic positive control soil was sampled from the Beachgrove (BG) experimental site located on the Bush Estate, *ca.* 15 km south of Edinburgh (NT243628). This site had previously been reported to show high nitric oxide fluxes as a result of nitrification (Thorman, 2002), approximately 10 times the rates measured at the Sourhope field site. The soil at the Beachgrove site was represented by two soil series, both of which were imperfectly drained brown earths and characteristic of a large area of the mixed agricultural land in the Midland Valley of Scotland (Vinten *et al.*, 2001).

### 5.2.2. Treatment structure

Soils were re-packed as described in Chapter 2 to a soil WFPS of 60% (average Sourhope soil moisture content) and maintained at 15°C throughout the incubation. Briefly; soils were re-packed to a soil bulk density of 1 g cm<sup>-3</sup> with ammonium nitrate (120 kg N ha<sup>-1</sup> yr<sup>-1</sup>) and de-ionised water applied to the sieved dry soil as a fine spray prior to re-packing. The autotrophic inhibitor DCD was also applied in the ammonium nitrate de-ionised water solution at a rate of 2mg DCD 100 g dry weight soil<sup>-1</sup>. The heterotrophic inhibitor cyclohexamide was applied as a fine powder prior to re-wetting due to health and safety risks involved with spraying this as a solution. The cyclohexamide was applied at 10 mg g soil<sup>-1</sup>, with 150 g dry weight of soil per microcosm.



The fluxes of nitric and nitrous oxide were determined from measurements made one and four days after addition of specific inhibitors. Nitric and nitrous oxide were measured using the dynamic and static chamber methods described in Chapter 2. Nitrous oxide was measured using ECD gas chromatography and nitric oxide by chemiluminescence.

*Table 4. Treatment applications made and processes inhibited by them.*

Treatment	Process inhibited
No inhibitor	none
DCD	autotrophic nitrification
Cyclohexamide	heterotrophic nitrification
Cyclohexamide + DCD	auto/heterotrophic nitrification

### 5.2.3. Nitrification rates

Nitrification rates were determined for each soil microcosm using  $^{15}\text{N}$  pool dilution techniques in combination with the specific inhibitors DCD and cyclohexamide. Isotopically labelled  $^{15}\text{N}$  nitrate was applied to soils as  $\text{NH}_4\text{NO}_3$  at 50 atom %  $^{15}\text{N}$  three days prior to the end of the experiment (previous measurements with 99 atom %  $^{15}\text{N}$  required dilution for CF IRMS analyses); destructive samples were analysed for  $^{15}\text{N}$  nitrate and ammonium immediately after  $^{15}\text{N}$  additions. Nitrate and ammonium were extracted from soils following the methods described in Chapter 2.

Following the calculations of Barraclough and Puri (1995) gross nitrification rates were determined from dilution of  $^{15}\text{N}$  labelled nitrate with time following oxidation of the  $^{14}\text{N}$  labelled soil ammonium pool (Equation 11).

Equation 11. Gross nitrification rate calculations of Barraclough and Puri (1995)

$$\text{Atom \% NO}_3^- \text{ tx} = \frac{\text{Atom \% NO}_3^- \text{ to}}{(1 + \Delta [\text{NO}_3^-] / [\text{NO}_3^-]_{\text{to}})^n}$$

n = gross nitrification rate ( $\mu\text{g N g}^{-1} \text{ d}^{-1}$ )

to = time zero sample point

tx = time x sample point

After three days of  $^{15}\text{N}$  incubation the soil microcosms were then analysed for nitric and nitrous oxide and gas samples were taken for  $^{15}\text{N}_2\text{O}$  analyses using the NERC stable isotope facility at CEH Merlewood.

## 5.3. Results

### 5.3.1. Nitric and nitrous oxide flux inhibition

The results from soil microcosm nitric and nitrous oxide flux are shown in Figure 38 a and b. Both the nitric and nitrous oxide flux were lower in DCD treated microcosms than non-inhibited soils. Maximum inhibition of nitric and nitrous oxide flux occurred in the Beachgrove soil with addition of DCD when compared with similar treatments in the Sourhope soil microcosms. The flux of nitrous oxide decreased from  $9 (\pm 1.1)$  to  $1.6 (\pm 1) \mu\text{g N}_2\text{O-N g soil}^{-1} \text{ h}^{-1}$  in Beachgrove control and DCD inhibited soil microcosms respectively. The flux of nitrous oxide was significantly ( $p < 0.0001$ ) greater in the Beachgrove soil than the Sourhope soil microcosms, even in the Beachgrove DCD inhibited microcosms.

The addition of DCD to control soil microcosms resulted in net uptake of nitrous oxide and more so with addition of cyclohexamide (Figure 38 a), although the inhibition was not significant relative to non-inhibited soil columns due to very large SE for almost all treatments.

The flux of nitrous oxide in control, lime, nitrogen and nitrogen plus lime treated columns were not similar to microcosm studies from previous work, where nitrogen treatments resulted in a significantly greater flux than control or lime

treatments alone (Chapters 6 and 7). The addition of cyclohexamide and DCD in combination to nitrogen and control soils decreased the inhibition of nitrous oxide flux relative to DCD or cyclohexamide treated microcosms alone (Figure 38 a), with nitrogen treatments the combination of both DCD and cyclohexamide in fact resulted in net emission of nitrous oxide compared with net uptake when applied individually.

The net uptake of nitrous oxide when inhibitor was applied alone could be due to increased nitrous oxide respiration as a result of either nitrifier denitrification or denitrification, although there was very large variation between samples as indicated by large standard errors.

When both autotrophic and heterotrophic nitrification were inhibited there was a net emission of nitrous oxide in the nitrogen treatment compared with addition of either inhibitor alone, this could be due to increased production via heterotrophic bacteria, for example *Paracoccus denitrificans*, which has both AMO and hydroxylamine oxidoreductase type enzymes and therefore able to produce nitrous oxide (Ferguson, 1998 and Zumft, 1997).

The effects of inhibitor on nitric oxide fluxes were not significant in all DCD amended soil microcosms ( $p = 0.1$ ) (Figure 38 b), the maximum inhibition of nitric oxide flux was in the Beachgrove soil which decreased from  $16 (\pm 4.7)$  to  $7 (\pm 2.7) \times 10^{-6} \mu\text{g NO-N g soil}^{-1} \text{ h}^{-1}$ .

Addition of DCD to lime treatments did not result in any inhibition of nitric oxide flux, however; in combination with nitrogen, nitric oxide flux decreased from  $8.1 (\pm \text{S.E. } 0.36)$  to  $5.4 (\pm \text{S.E. } 0.14) \times 10^{-6} \mu\text{g NO-N g soil}^{-1} \text{ h}^{-1}$ .

Changes in the soil ammonium and nitrate pools were measured after 14 days of microcosm incubation when the columns were destructively sampled (Figure 38 c).

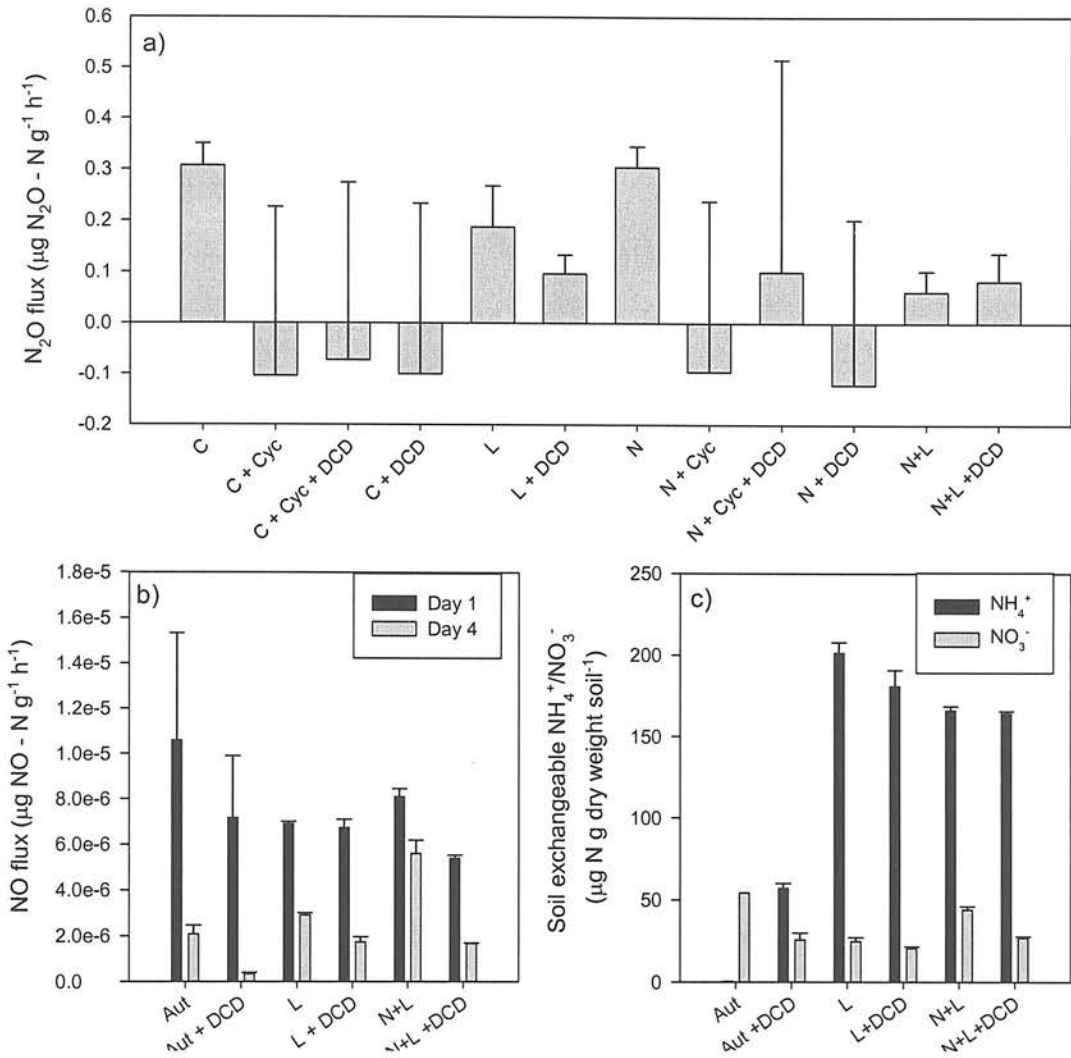


Figure 38. The effects of DCD and cyclohexamide on nitric and nitrous oxide flux in soil microcosms. Nitrous oxide flux was measured on day one following treatment and inhibitor additions. The soil exchangeable N results are presented from soil microcosms extracted at the end of the microcosm incubation after 14 days (C – control; N –  $\text{NH}_4\text{NO}_3$ ; L – Lime; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; Aut – Beachgrove soil; Cyc – cyclohexamide; DCD – dicyandiamide)

Following 14 days of inhibition the soil ammonium pool in the Beachgrove soil without DCD had decreased from approximately  $200 \mu\text{g N g}^{-1}$  dry weight soil to  $0.5 \mu\text{g N g}^{-1}$  dry weight soil which implies that the net rate of nitrification was approximately  $7.7 \mu\text{g N g}^{-1}$  dry weight soil  $\text{h}^{-1}$ . The addition of DCD inhibited the rate of nitrification in the Beachgrove soil by 96% to  $0.45 \mu\text{g N g}^{-1}$  dry weight soil  $\text{h}^{-1}$ . The addition of DCD to lime and nitrogen plus lime treatments did not significantly inhibit the reduction of ammonium compared with uninhibited soils (Figure 38 c). Addition of DCD to lime treated soils actually increased the net nitrification rates.

### 5.3.2. Effects of specific inhibitors on gross rates of nitrification and $^{15}\text{N} - \text{N}_2\text{O}$ enrichment

Following pool dilution with  $^{15}\text{N}$  labelled nitrate the gross nitrification rates in Figure 39 d show that addition of DCD to lime treated columns increased the nitrification rates compared with net rates determined from changes in the soil ammonium pool alone.

Overall the effects of inhibitors on the gross nitrification rates are unclear with increased rates following additions of DCD to control, lime and nitrogen plus lime treatments (Figure 39 d.). Overall gross rates of nitrification were in agreement with those of other studies in similar soils ranging from  $0.6$  to  $1.2 \mu\text{g N g}^{-1} \text{d}^{-1}$  in control and lime plus DCD incubations respectively (Davidson *et al.*, 1972 and Barraclough and Puri, 1995). With exception of the nitrogen incubation treatment the autotrophic nitrification inhibitor resulted in increased gross nitrification rates.

The isotopic composition of the nitrous oxide produced as a result of  $^{15}\text{N}$  labeled nitrate additions are shown in Figure 39 c, addition of inhibitors actually increased the  $^{15}\text{N}$  enrichment of nitrous oxide relative to uninhibited microcosms.

The relative contribution of nitrification and denitrification to total  $\text{N}_2\text{O}$  production was calculated from the  $m/z$  values 44/45/46 measured using GC/CF IRMS and concentrations of nitrous oxide determined from ECD gas

chromatography. Less than 1% of total nitrous oxide flux was via denitrification (calculated from masses 45 and 46) and more than 99% of the total nitrous oxide flux was produced via nitrification and oxidation of non labeled ammonium although there were no significant treatment effects.

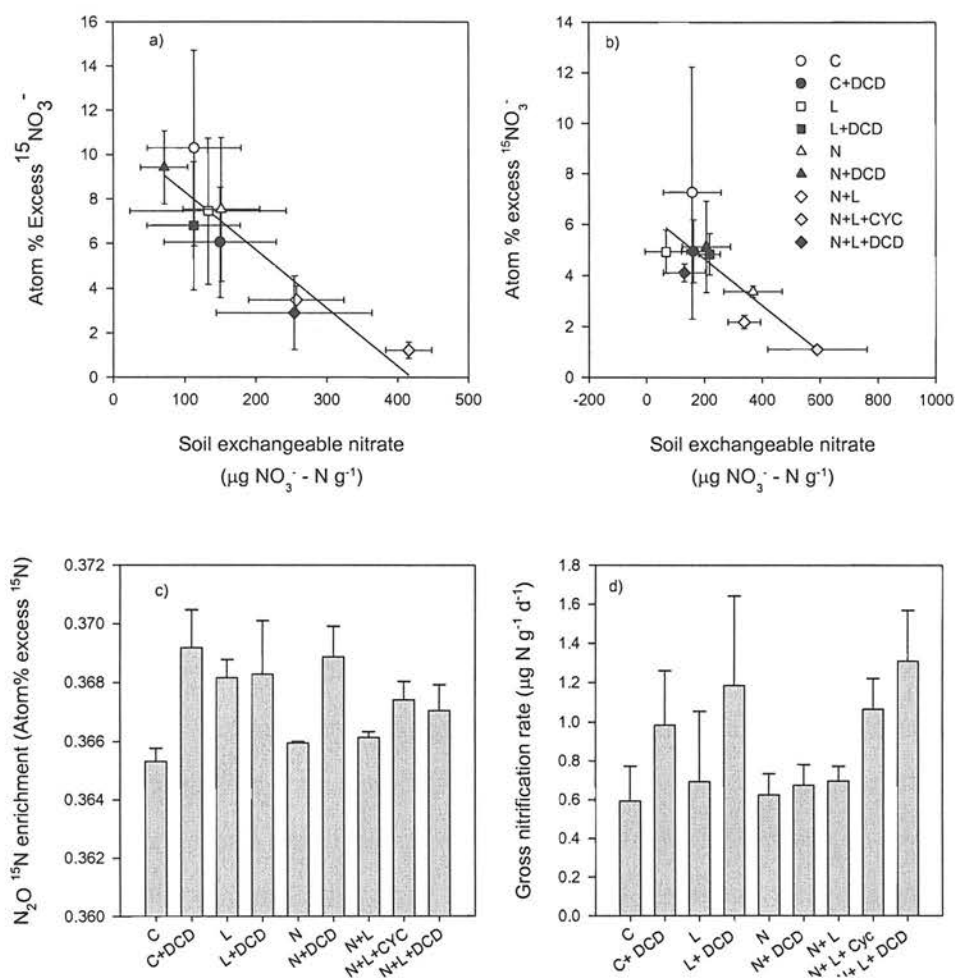


Figure 39. Changes in soil nitrate concentration and  $^{15}\text{N}$  enrichment following pool dilution at incubation  $t_{3d}$  (a) and  $t_{0d}$  (b) after two weeks of microcosm inhibition. The effects of inhibitor on the  $^{15}\text{N}$  enrichment of nitrous oxide (c) and gross nitrification rates determined from pool dilution equations (d) (C – Control; L – Lime; N –  $\text{NH}_4\text{NO}_3$ ; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; Cyc – cyclohexamide; DCD – Dicyandiamide)



The soil exchangeable nitrate from the pool dilution results were significantly correlated with the atom % excess  $^{15}\text{N}$  values of nitrate. Figure 39 a shows the correlation between soil nitrate concentration and atom % excess  $^{15}\text{N}$  nitrate after 3 days incubation ( $R^2 = 0.84$ ,  $p < 0.001$ ) and Figure 39 b that for time zero incubation ( $R^2 = 0.65$ ,  $p = 0.009$ ).

The effects of inhibitor on this correlation whilst not significant do show that DCD results in a  $^{15}\text{N}$  depletion of the soil nitrate pool for control, nitrogen and nitrogen plus lime treatments; whereas the lime treated soils with DCD were enriched with  $^{15}\text{N}$  relative to the uninhibited lime treatments.

## 5.4. Discussion

The results from both gaseous fluxes and changes in soil exchangeable ammonium show that the rates of nitrogen cycling in the acidic podzolic soils from the Sourhope treatments have very low rates of nitrification compared with those from the autotrophic Beachgrove soil.

Following the addition of lime and nitrogen to control plots in the field since 1998 the soil pH was measured in soil sampled from the different treatments prior to these experiments. The four Sourhope soil treatments of control, lime, nitrogen and nitrogen plus lime had a soil pH of 4.76, 5.6, 5.16 and 5.24 respectively; long term fertilisation with ammonium based fertilisers and lime has already been shown to result in decreased and increased soil pH respectively (Parkin *et al.*, 1985). Low soil pH has also been shown to favour heterotrophic nitrifiers more than autotrophic nitrifiers that have an optimum pH of 7.8 (Hagopian and Riley, 1998).

The results from these studies showed a reduction in the nitrous oxide emission in lime amended soils relative to control soils without inhibitor, by 37% in the lime only and 80% in the nitrogen and lime treatments. Since the  $^{15}\text{N}$  labelling of the nitrous oxide produced indicated that more than 99% was due to nitrification it would seem that decreased emissions as a result of liming are due to changes in the nitrifier rather than denitrifier populations.

Other studies have also linked decreased nitrous oxide emissions with liming and pH effects to changes in nitrifier populations, Borken and Brumme (1997) noted significant decreases in the nitrous oxide emissions relative to non limed soil by upto 74% in acidic forest soils, whilst Yamulki *et al.* (1997) measured increasing emissions of nitrous oxide with increasing soil pH associated with regular liming since 1903 in the Park Grass experiment at the Rothamsted Experimental Station, they also noted increased NO emissions in more acidic than alkaline pH soils as a result of chemodenitrification.

These data further re-iterate that the time scale over which microbial populations change is also an important factor when looking at treatment effects of pH on N transformations, this study correlated decreased nitrous oxide emissions with increasing soil pH whilst the study of Yamulki *et al.* (1997) noted the opposite, the study of Yamulki *et al.* (1997) was carried out on a grassland soil which had received lime and fertiliser amendments since 1903 and 1856 respectively whilst these study soils have only received lime and fertilizer since 1998 when the Soil Biodiversity Programme was established.

These contrasting results may reflect the diversity and stability of ammonium oxidizer populations, Gray *et al.* (2003) measured changes in soil AOB populations in the Sourhope soil with time following lime amendments, they noted that lime had the greatest affect on the community structure of autotrophic ammonium oxidizers resulting in increased heterogeneity with time and also increased ammonium oxidizer potential rates when compared with control unamended soils. Given that autotrophs prefer neutral to alkali pH and that liming an acidic soil results in increased soil pH, it is perhaps an increase in pH which creates more favourable conditions for autotrophic growth on ammonium (Prosser, 1989) and therefore increased ammonium oxidation coupled to AOB population changes compared with the more acidic control soils.

There were no significant differences in the gross nitrification rates between the uninhibited treatments relative to the control treatment (Figure 39 d); the addition of DCD and or cyclohexamide did result in increased gross nitrification rates relative to the comparable uninhibited treatments (Figure 39 d). The hole in the pipe model of

Firestone and Davidson (1989) would predict greater losses of both nitric and nitrous oxide with increases in the rates of nitrification; however, both nitric and nitrous oxide emissions actually decreased in DCD and cyclohexamide amended treatments compared with uninhibited treatments (Figure 36 a and b).

With inhibition of either autotrophic or heterotrophic nitrification using DCD or cyclohexamide respectively, competition for ammonium is reduced. The autotrophic nitrifiers have been shown to have lower  $K_m$  values for ammonium than heterotrophic nitrifiers (Stark and Firestone, 1996) (k and r strategists respectively), these data show that in the treatments without DCD gross nitrification rates were lower and autotrophic nitrifiers more active, conversely in DCD amended treatments where autotrophs were inhibited, heterotrophs resulted in increased gross nitrification rates.

The inhibition of autotrophic nitrification allows heterotrophs to compete for ammonium and therefore with higher  $K_m$  values they also have greater  $V_{max}$  and therefore rates of nitrification increased with inhibition of autotrophic nitrifiers.

The nitric and nitrous oxide data would also suggest that heterotrophic nitrification in these experiments results in lower emissions of these two gases from the soil, in which addition of DCD decreased the emission of nitrous oxide although these were not significantly different to control soils. However there were large standard errors associated with nitrous oxide emissions from the inhibited soils and would therefore indicate that the effects of inhibitors were not completely effective throughout the soil column. Heterotrophic nitrification may also be carried out by the aerobic denitrifier *Thiosphaera pantropha* which produces nitrite from ammonia which would not be affected by either DCD or cyclohexamide and may account for reduced nitrous oxide emissions in inhibited soil treatments (Robertson and Keunen 1984).

In pure cultures of *Nitrosomonas europaea* and *Alcaligene faecalis* an autotrophic and heterotrophic nitrifier respectively, Anderson *et al.* (1993) measured nitric and nitrous oxide emissions, with 10 fold more nitrous oxide produced per cell by *A. faecalis* than *N. europaea*,

The use of inhibitors to partition the activities of heterotrophic and autotrophic micro-organisms are associated with many difficulties. Firstly, it is very difficult to achieve uniform distribution of inhibitors, and it is likely that microsites remain in the soil which are unaffected by their application, allowing the activity of micro-organisms to continue (Castaldi and Smith, 1990), which is clearly shown with the large standard error in nitrous oxide measurements. Improvements in the application of specific inhibitors by using more vigorous mixing and smaller soil particle sizes could help to improve their efficacy once re-packed in the soil columns. Secondly the inhibition of a group of micro-organisms can result in the behaviour of those organisms that remain unaffected by the inhibitor to be different from their activity in the unamended soil (Wrage *et al.* 2004). The gross rates of nitrification were greater in the inhibited soils than in their uninhibited analogues which are a reflection of potential rather than actual process rates *in situ*.

These data show that land management treatments used in this study have an affect on the production of nitric and nitrous oxide which may be mediated via changes in soil chemistry as measured by soil pH. Increasing the pH had little effect on the gross nitrification rates but did result in decreased nitrous oxide emissions. Following the use of the inhibitor DCD and  $^{15}\text{N}$  pool dilution techniques, gross nitrification rates increased in all soil treatments at all soil pH's with the exception of the nitrogen treatments; this indicates that heterotrophic nitrification rates are greater than autotrophic nitrification rates in these soils but are out competed by autotrophs for ammonium in uninhibited soils. With no effect of nitrogen addition on the gross nitrification rates between DCD and non DCD inhibited soils would indicate that either autotrophs are not nitrifying in these soils or that DCD had no effect in this treatment. With increased emissions of nitrous oxide in the nitrogen and control treatments compared to lime and nitrogen plus lime treatments possibly heterotrophic nitrification can result in higher emissions than autotrophic.

## **Chapter Six. Long term incubation of soil microcosms under different land management regimes.**

### **6.1. Introduction**

#### **6.1.1. Land management and nitrogen cycle**

Due to the anthropogenic impacts on nitrogen losses to the atmosphere as nitric and nitrous oxide and nitrate leaching, the cycling of nitrogen has in recent times become of greater environmental significance.

The influence of land management regimes, which involve supplementation of natural nitrogen pools with fertilizer additions on microbial processes is increasingly well studied both in the lab and *in situ* (Clayton *et al.*, 1994; Hutsch *et al.*, 1994; Bouwman 1996; Abbasi and Adams, 2000), although studies relating the influence of land management with microbial diversity have not been carried out extensively (Torsnik, 1998; Mendum *et al.*, 1999; Marschner *et al.*, 2003; Gray *et al.*, 2003; Grayston *et al.*, 2004). It is not clear how land management will affect microbial molecular diversity and in turn these effects on microbially mediated nitrogen cycling. This has implications for worldwide carbon and nitrogen cycling and greenhouse gas emissions; if land management affects the molecular diversity of important functional groups, for example ammonium oxidizers, this could result in a loss of function and either an increase or decrease in the nitric or nitrous oxide production which could depend upon the functional diversity of other species of bacteria.

Research has been directed at quantifying the biodiversity of microbial populations in terrestrial ecosystems (Ward *et al.*, 1990; Pankhurst *et al.*, 1996; Brussard *et al.*, 1996), however very little work has been done to try and link this microbial diversity with microbial functioning (Torsvik, 1998; Avrahami *et al.*, 2002). The links between microbial diversity and function are often difficult to precisely quantify, this is largely due to the large molecular diversity in soils and relatively low numbers of species isolated and identified in pure culture (Ward *et al.*, 1990).



Recent developments in soil microbial ecology have used long-term microcosm incubations to select for specific functional groups of microorganisms in soil. Following nitrogen and carbon additions,  $^{13}\text{C}$  labeled compounds were applied to label microbial DNA and RNA with heavy carbon (Radajewski *et al.*, 2000), thus allowing both function and diversity to be linked via extraction of  $^{13}\text{C}$  enriched DNA and RNA from soils indicative of active functioning populations. Different phylogenetic probes may then be used to identify the “heavy”  $^{13}\text{C}$  enriched DNA extracted from soils and separated using CsCl density gradient centrifugation, and indeed these techniques have already been used to identify active components of autotrophic ammonium oxidizer communities (Whitby *et al.*, 2001).

It is interesting to note that 80% of ecosystem function is governed by only 20% of the population and is known as Pareto’s Law; this was first used to describe economic growth and was adapted to mathematically describe function and diversity relationships in biological systems (Reed and Hughes, 2002). The 80:20 law in which fewer individuals are responsible for the majority of ecosystem function, increases the importance of studies linking both function and diversity with the potential for multi-cycle functional groups able to switch function between carbon and nitrogen cycles, as is the case with methanotrophs and ammonium oxidizers (Mandernack *et al.*, 2000). This versatility will provide the ecosystem resilience required in the face of increasing environmental stress, allowing carbon and nitrogen flows to be maintained and regulated.

In addition to the land management treatments applied in the field, further treatments with nitrite and acetate were used to complement previous molecular work with enrichment cultures set up using Sourhope soil, the enrichment cultures used nitrite and acetate to enrich for nitrite oxidisers to develop phylogenetic probes for use in the field. Soil microcosms were used to measure nitric and nitrous oxide emissions and both conventional molecular approaches with 16S rRNA probes and also new  $^{13}\text{C}$  DNA/RNA extraction techniques were used to quantify changes in the microbial populations from the soil incubations (Radajewski *et al.*, 2000). Addition of nitrogen and lime in conjunction with readily available carbon sources have been



shown to change the dominance of organisms capable of utilising those substrates (Ward *et al.*, 2003), however it is not known exactly how land management regimes will affect the processing of nitrogen and nitric and nitrous oxide flux in relation to effects on microbial diversity and the links between the two.

To test the hypothesis that addition of lime and nitrogen to Sourhope control soil could result in increased nitric and nitrous oxide emissions mediated via a change in the microbial functional diversity and a switch from heterotrophic to autotrophic nitrification, a continuous flow system was developed. This continuous flow system was used to label microbial populations with  $^{13}\text{C}$  labeled  $\text{CO}_2$  following incubation of soil with the Sourhope land management treatments of nitrogen, lime, nitrogen plus lime, and control. The  $^{13}\text{C}$  pulse labeling was used to link changes in diversity with function (measured as changes in nitric and nitrous oxide production and gross nitrification rates) of microbial nitrifying populations.

## 6.2. Materials and Methods

### 6.2.1. Experimental set-up

Soil was sampled from the Sourhope destructive control plots as 20cm by 20cm by 10cm depth soil monoliths. The litter layer was removed and the soil monoliths were then broken up, sieved (10mm) and dried at 21°C in a temperature controlled drying oven (Apex, UK); to a gravimetric soil water content below 0.313 g  $\text{H}_2\text{O}$  g $^{-1}$  soil. The soil was then bulked and mixed thoroughly and stored at 5°C until required.

Soil (229-g dry weight of soil) was re-packed into microcosms following addition of de-ionised water to achieve a gravimetric soil moisture content of 0.313 g  $\text{H}_2\text{O}$  g $^{-1}$  soil (WFPS of 50%). The soil microcosms were fitted with a fine plastic mesh end cap (1mm weave) and silanized glass wool (Supelco Inc., USA) at the base

to allow leaching through the soil columns following treatment addition but not loss of soil.

The treatments are summarized in Table 5 and included those applied at the field site; control, nitrogen ( $\text{NH}_4\text{NO}_3$ ), lime applied as  $^{13}\text{C}$  labelled  $\text{Ca}^{13}\text{CO}_3$  (99 atom % excess  $^{13}\text{C}$ ) and nitrogen plus lime. Further treatments were applied and included nitrite applied as sodium nitrite, nitrite plus  $^{13}\text{C}$  double labelled acetate, ( $^{13}\text{CH}_3^{13}\text{CO}_2\text{Na}$ ) at 99 atom % excess  $^{13}\text{C}$ ; nitrogen plus acetate; control plus acetate and a second control treatment with  $^{12}\text{C}$  labelled  $\text{CO}_2$ . Nitrite was applied to enrich for nitrite oxidizer populations within the soil to help further develop a universal 16S rRNA primer for those organisms; acetate was added as a simple carbon source to select for heterotrophic and denitrifying bacteria. The treatments were applied at three levels of N and C addition which were equivalent to one week, six months and six week addition relative to those in the field. Treatments were applied in weekly rainfall equivalents of 39.78 ml de-ionised water per column (equivalent to the Sourhope yearly average rainfall of  $17.5 \text{ mm week}^{-1}$ ).

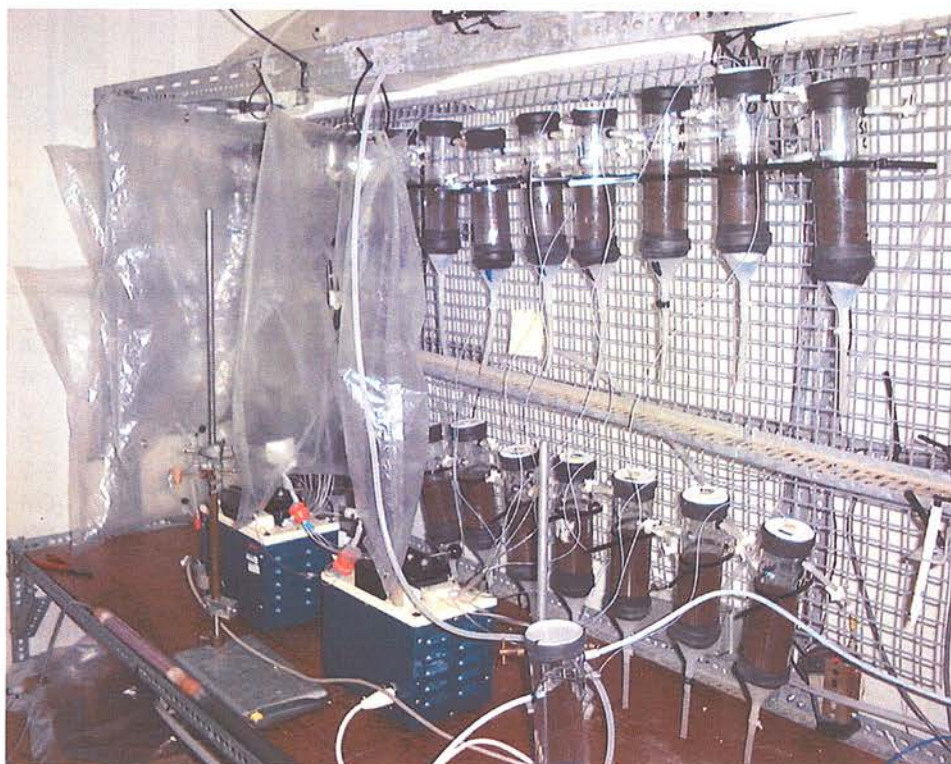
Dynamic soil microcosms were modified from those described in Chapter 2 so that a flow through system could be employed for each column. Artificial air was made using compressed air with a certified  $\text{CO}_2$  concentration of less than 10 ppm in Tedlar bags (BOC, UK), this was amended with either  $^{13}\text{C}$  or  $^{12}\text{C}$  labeled  $\text{CO}_2$  (BOC Special Gases, UK; CK Gases, USA). Each 25 l Tedlar bag was filled with this low  $\text{CO}_2$  air and then  $^{13}\text{C}$  labelled  $\text{CO}_2$  (CK gases, USA) was injected via rubber septa directly into the bag to give a final  $\text{CO}_2$  concentration of 7000 ppm at 99 atom %  $^{13}\text{C}$ .

*Table 5. Summary of treatment additions to soil microcosms, control treatments received de-ionised water only.*

Treatment	Treatment addition rates (mg of compound column <sup>-1</sup> )	
	One week additions	6 month additions
Control 1 (C)	-	-
Nitrogen (NH <sub>4</sub> NO <sub>3</sub> )	0.42	10.92
Lime (Ca <sup>13</sup> CO <sub>3</sub> ) (L)	10.54	274.04
N + L	0.42 and 10.54	10.92 and 274.04
C + Acetate ( <sup>13</sup> C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> Na) ( <sup>13</sup> Act)	0.21	5.46
N + <sup>13</sup> Act.	0.42	10.92 and 5.46
N + L + <sup>13</sup> Act.	0.42 / 10.54 / 0.21	10.92 / 274.04 / 5.46
Nit (NaNO <sub>2</sub> )	0.45	11.7
Nit + <sup>13</sup> Act.	0.45 and 0.21	11.7 and 5.46
Control 2 (no $\delta^{13}\text{CO}_2$ )	-	-

The control two treatment air was made up using <sup>12</sup>CO<sub>2</sub> instead of <sup>13</sup>C labelled CO<sub>2</sub>. The two air mixtures were pumped through the soil columns using peristaltic pumps fitted with 0.6 ml min<sup>-1</sup> pump tubing (Gradko Int. Ltd.), this flow rate replaced the volume available for gas transport within the column approximately every 8.3 hours.

The soil microcosms were arranged in a randomised block design (Figure 40), PTFE tubing was used to supply artificial air to each soil microcosm from the peristaltic pumps which are shown in Plate 5.



*Plate 5. Soil microcosm set-up for long term soil incubation and  $^{13}\text{C}$  pulse labeling.*

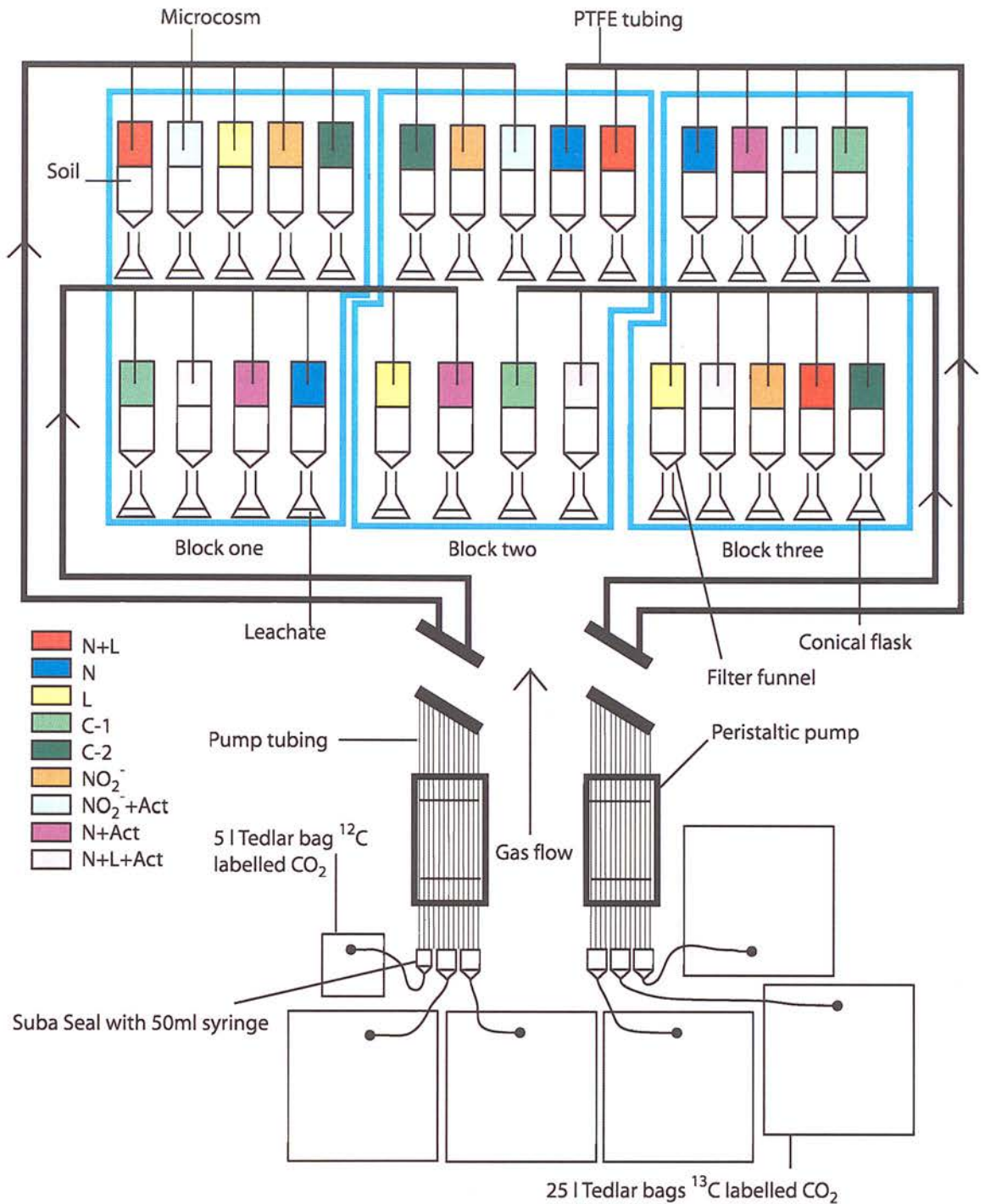


Figure 40. Schematic diagram of long term enrichment microcosm set-up.



### 6.2.2. Microcosm CO<sub>2</sub> labeling

The concentration and flow rate of CO<sub>2</sub> required to label the soil nitrifiers was determined using experimentally determined respiration rates and published pure culture work.

Initial soil respiration rates were determined from measurements of CO<sub>2</sub> fluxes in re-packed soil columns following addition of inorganic N prior to set up of the experiment. The soil respiration rate was determined using static chamber methods and gas samples were analysed for CO<sub>2</sub> using a GC fitted with an FID.

The potential CO<sub>2</sub> required by autotrophic nitrifiers of both ammonium and nitrite was determined from pure culture work already published (Prosser, 1989). Since autotrophs use inorganic nitrogen as an energy source and CO<sub>2</sub> as a carbon source, every molecule of CO<sub>2</sub> incorporated into the biomass of an autotrophic ammonium oxidising bacteria (AOB) requires 35 molecules of NH<sub>4</sub><sup>+</sup> to provide the energy for this incorporation. The nitrite oxidising bacteria (NOB) require 85 to 115 molecules of NO<sub>2</sub><sup>-</sup> to provide the energy for incorporation of one molecule of CO<sub>2</sub>. From these published data it was possible to determine the total CO<sub>2</sub> required using Equation 12, the ratio 115/35 represents the nitrite reduced following the nitrification of ammonium which is used by the nitrite oxidising bacteria for CO<sub>2</sub> assimilation.

Equation 12. CO<sub>2</sub> requirement for autotrophic nitrification

$$\text{CO}_2 \text{ required for autotrophic nitrification} = (\text{CO}_2 \text{ for AOB}) + ((\text{CO}_2 \text{ for AOB}) * 115/35)$$

An application rate of 0.42 mg NH<sub>4</sub>NO<sub>3</sub> column<sup>-1</sup> week<sup>-1</sup> requires 0.15 μ moles CO<sub>2</sub> column<sup>-1</sup> week<sup>-1</sup> for AOB from Equation 12 and 0.493 μ moles CO<sub>2</sub> column<sup>-1</sup> week<sup>-1</sup> for NOB from Equation 13.



Equation 13. Moles of CO<sub>2</sub> utilised for autotrophic ammonium oxidation

$$\text{Moles CO}_2 (\text{AOB}) = \frac{5.25 \mu \text{ moles NH}_4 \text{ column}^{-1} \text{ week}^{-1}}{35 \text{ moles CO}_2}$$

Equation 14. Moles of CO<sub>2</sub> utilised for autotrophic nitrite oxidation

$$\text{Moles CO}_2 (\text{NOB}) = \text{Moles CO}_2 (\text{AOB}) \times 3.287$$

The total number of moles of CO<sub>2</sub> incorporated via autotrophic nitrification at the one week addition rate was 0.643  $\mu$  moles CO<sub>2</sub> column<sup>-1</sup> week<sup>-1</sup> which is equal to 28.9  $\mu$ g CO<sub>2</sub> column<sup>-1</sup> week<sup>-1</sup>.

Soil carbon dioxide production in microcosms incubated at 15°C and 50% WFPS was determined using FID gas chromatography and flow through soil microcosms, in which a constant flow of “zero” air (40ml min<sup>-1</sup>) up through the soil column was sampled in a modified microcosm. Samples were taken after 5, 10, 15 and 30 minutes using 10ml gas tight syringes after switching in line with the flow through system.

### 6.2.3. Nitric and nitrous oxide flux measurements

The flux of nitric and nitrous oxide was determined following initial treatment additions. The soil microcosms were flushed with compressed air (BOC, UK) prior to analysis using the dynamic chamber system described in Chapter 2 for NO measurements and static chamber methods for N<sub>2</sub>O measurements.

Microcosm flushing was necessary due to the build up of both nitric and nitrous oxide during column enrichment as a result of the closed incubation system used to maintain a <sup>12</sup>C free CO<sub>2</sub> system.

Initial results indicated that the one week equivalent treatment additions did not significantly increase the production of nitric or nitrous oxide and therefore 6 month equivalent treatment additions were made 2 weeks following initial enrichment.

#### 6.2.4. Nitrification and $^{15}\text{N}$ pool dilution

Gross nitrification rates were calculated using equations from Barraclough and Puri (1995), fractionation factors and enrichment factors associated with nitrification and denitrification of ammonium and nitrate to nitrous oxide were calculated using two equations from Lajtha and Michener (1990)

Fractionation factors were calculated using the following:

$$\alpha = 1 + (\delta^{15}\text{N substrate}/1000) / 1 + (\delta^{15}\text{N product}/1000)$$

Enrichment factors were calculated using the following:

$$\varepsilon = \text{Atom}\%^{15}\text{N substrate} - \text{Atom}\%^{15}\text{N product} / 1 + (\text{Atom}\%^{15}\text{N product}/1000)$$

Fractionation factors were also used to determine enrichment factors:

$$\varepsilon = (\alpha - 1) * 1000$$

#### 6.2.5. Molecular sampling and methods

Molecular analysis involved the amplification of 16S rRNA genes from extracted nucleic acids using primers specific for eubacteria (heterotrophs),  $\beta$ -ammonia oxidizers and nitrite oxidizers.

Following nitric and nitrous oxide flux measurements 0-1cm depth sub samples were taken from each soil core and stored in Eppendorfs at  $-80^{\circ}\text{C}$  prior to RNA and DNA extraction. The 16S rRNA genes were analysed following PCR amplification with the specific primers followed by DGGE (denaturing gradient gel electrophoresis) and sequencing of the DGGE bands of interest. All molecular analyses were carried out by Dr Arjen Speksnijder at the University of Aberdeen.

### 6.3. Results

#### 6.3.1. NO and N<sub>2</sub>O flux

The effect of nitrogen addition at the one week application rates on the nitric and nitrous oxide flux are shown in Figure 41 a and b. Following initial treatment additions to the soil microcosms, nitrite resulted in a significant increase in both nitric and nitrous oxide flux ( $p < 0.05$ ) at  $0.014 \mu\text{g NO-N g soil}^{-1} \text{ h}^{-1}$  and  $0.0096 \mu\text{g N}_2\text{O-N g soil}^{-1} \text{ h}^{-1}$  respectively. The control soil microcosm fluxes for both nitric and nitrous oxide were lower ( $-0.414 \times 10^{-3}$  and  $0.048 \times 10^{-3} \mu\text{g NO-N and N}_2\text{O-N g soil}^{-1} \text{ h}^{-1}$  respectively) in comparison to all other treatment incubations. The nitric oxide flux following nitrite additions in these experiments were attributed to abiological reactions, determined from fluxes measured in sterile soil microcosms supplemented with sodium nitrite (Chapter 2).

Following the addition of treatments at low application rates, nitric and nitrous oxide production significantly ( $p < 0.05$ ) increased in microcosms to which nitrogen ( $0.147 \mu\text{g N}$ ) had been applied, there were no significant effects of blocking within the controlled temperature room following two way analysis of variance (Minitab, USA).

The treatment affects on flux of nitric and nitrous oxide were significant ( $p < 0.05$ ) and are summarized in Table 6 (Minitab, USA). The addition of acetate to nitrogen and nitrogen plus lime increased nitrous oxide flux relative to non acetate amended soils (Figure 41 a), whereas addition of acetate resulted in decreased nitric oxide flux relative to non acetate nitrogen and nitrogen plus lime treatments respectively (Figure 41 b).

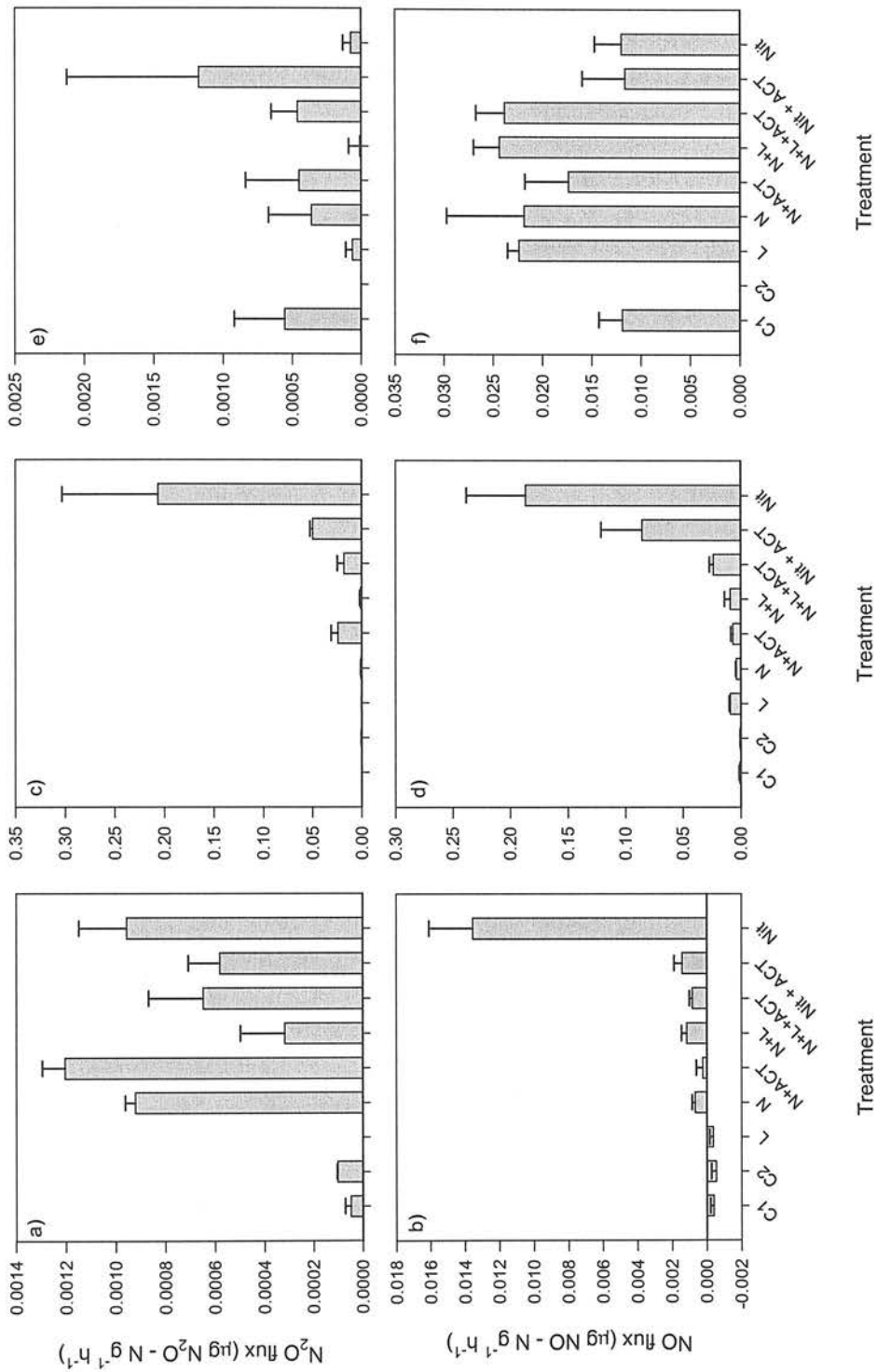


Figure 41. Nitric and nitrous oxide flux in soil microcosms following addition of treatments at 1 and 21 wk field equivalents a-b and c-d respectively. The effects of  $\text{NH}_4\text{NO}_3$  addition on nitric and nitrous oxide flux following 6 months of long term incubation e-f (C-1 – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate).

Table 6. Effects of one week treatment additions, ANOVA Tukeys pairwise comparison tests were determined for 95% confidence intervals (Minitab, USA). Statistically significant treatment differences are denoted by a \* for nitric oxide flux data and + for nitrous oxide flux data.

Treatment	Control 1	Control 2	Nitrogen	Lime	N+L	N+Act	N+L+Act	NO <sub>2</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup> + Act
Control 1	-----					+		+	
Control 2		-----				+		+	
Nitrogen			-----						
Lime				-----					
N+L					-----	+			
N+Acetate						-----			
N+L+Acetate							-----		
NO <sub>2</sub> <sup>-</sup>	*	*	*	*	*	*	*	----	
NO <sub>2</sub> <sup>-</sup> + Acetate								*	----

Since both nitric and nitrous oxide flux were relatively low following the one week treatment application rate, application rates were increased. The increased treatment additions were made on the 16/05/01, 4 weeks after initial treatment additions and were equivalent to 1/2 the annual field addition rate.

The flux of nitric and nitrous oxide were determined 2 days following the increased treatment additions, the results are shown in Figure 41 c and d. The

addition of nitrite resulted in the greatest fluxes of both nitric and nitrous oxide, 0.186 and 0.135  $\mu\text{g N g}^{-1} \text{h}^{-1}$  respectively, again due to abiological reactions.

Amendment of soil microcosms with acetate at the increased application rates resulted in increased nitric and nitrous oxide flux relative to nitrogen and nitrogen plus lime treatments alone, this was in contrast to the low application rates where acetate only increased the emissions of nitrous oxide compared with non-acetate amended treatments.

### **6.3.3. $^{15}\text{N}$ pool dilution of microcosms incubated with Sourhope treatments for 4 months. Gross nitrification rates and $^{15}\text{N}$ - $\text{N}_2\text{O}$ data.**

The addition of  $^{14}\text{NH}_4^{15}\text{NO}_3$  to determine gross nitrification rates following four months of treatment incubations resulted in increased nitric and nitrous oxide flux in all incubation treatments.

The results in Figure 41 e and f show that previous enrichment of microcosms with nitrogen enhanced nitric and nitrous oxide flux relative to control treatments following addition of  $^{15}\text{N}$  labelled  $\text{NH}_4\text{NO}_3$ , although these were not significant. However, enrichment with acetate and nitrogen plus lime and nitrite plus acetate increased nitrous oxide flux relative to both control and nitrite and nitrogen plus lime treatments alone (Figure 41 e and f).

There were no significant treatment effects on gross nitrification rates determined by  $^{15}\text{N}$  pool dilution (Figure 42). However, addition of acetate to nitrogen and nitrite treatments resulted in an increase in the gross nitrification rates compared with non acetate amended treatments and controls, addition of lime alone also resulted in increased gross nitrification rates (Figure 42).



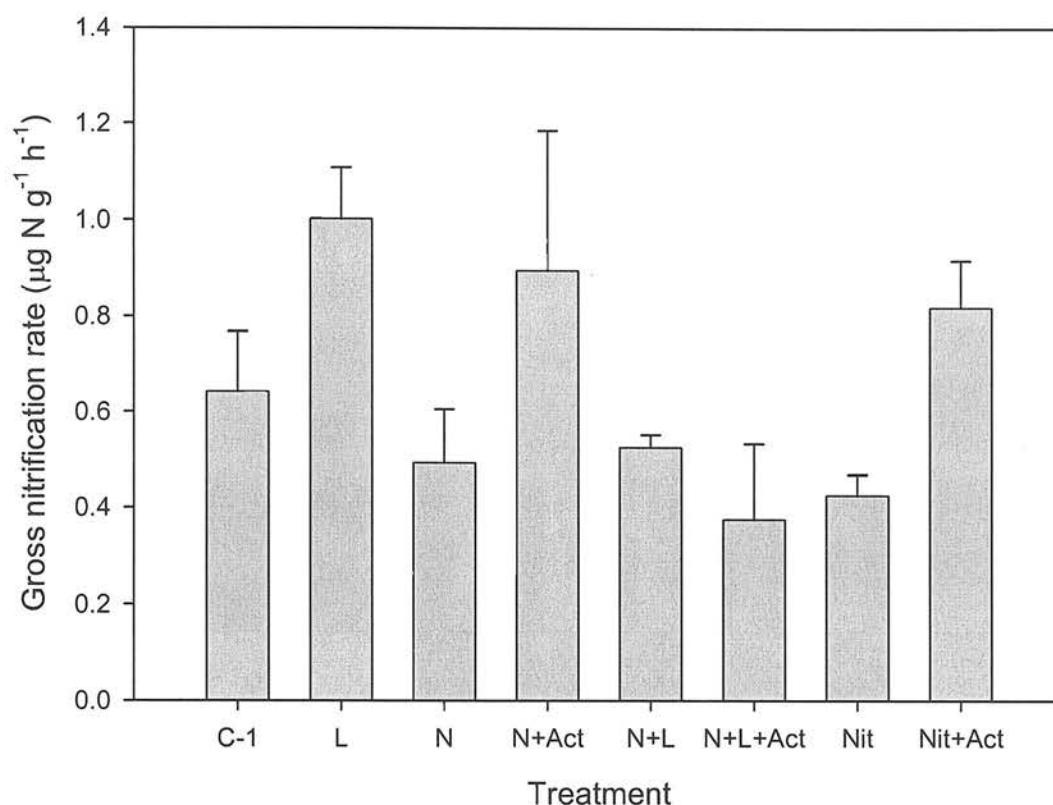


Figure 42. Gross nitrification rates in soil microcosms following  $^{15}\text{N}$  pool dilution. (C-1 – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate). (Error bars are  $\pm$  S.E.  $n=3$ )

The isotopic enrichment of nitrous oxide and di-nitrogen were analysed using CF-IRMS (CEH Merlewood) following  $^{15}\text{N}$  pool dilution incubation, however these did not result in production of  $^{15}\text{N}$  di-nitrogen gas. The isotopic ratio of nitrous oxide produced following addition of  $\text{NH}_4^{15}\text{NO}_3$  are shown in Figure 43 the initial isotope ratios for soil nitrate and ammonium applied to the soil microcosms following KCl extraction were 12.454 and 0.432 atom % excess  $^{15}\text{N}$  respectively.

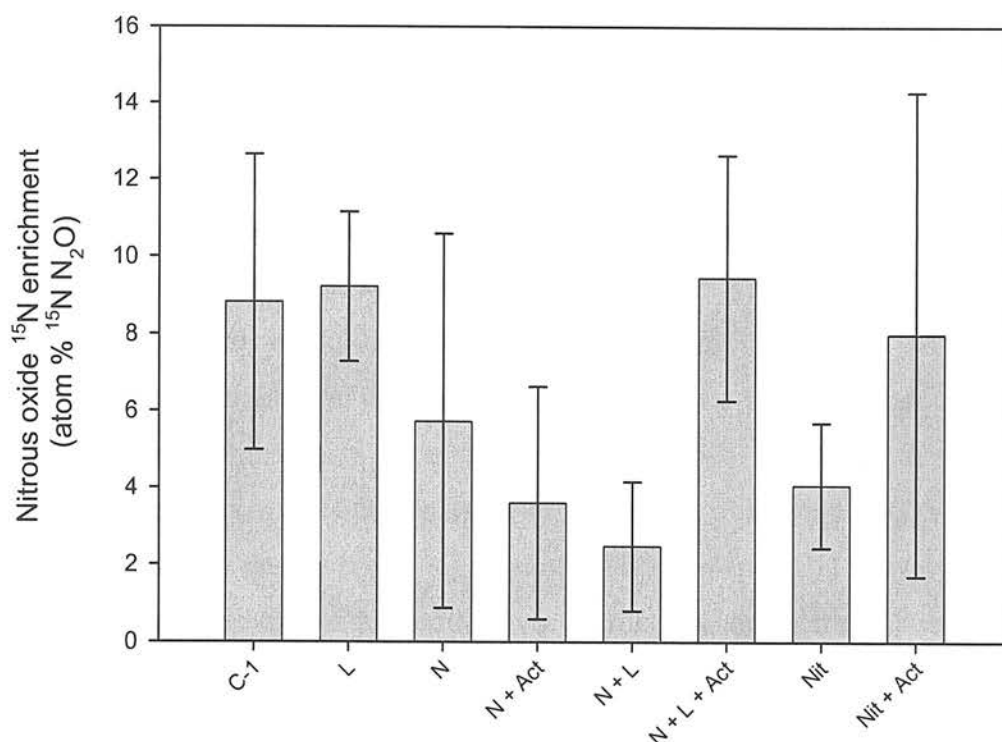


Figure 43. Isotopic composition of nitrous oxide produced following addition of  $^{15}\text{N}$  labelled  $\text{NO}_3^-$  (99 atom % excess) as  $\text{NH}_4\text{NO}_3$  to soil microcosms after 6 months of treatment enrichment (C-1 – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate).

The distribution of N atoms within the nitrous oxide molecules were determined using equations from Stevens and Laughlin (1998). The proportion of nitrous oxide as masses 45:46 and 46:44 were determined from m/z values, if the atom%  $^{15}\text{N}$  calculated using 45/46 is not significantly different from that calculated using 46/44 then the distribution of  $^{15}\text{N}$  is random and therefore there is only one source of nitrous oxide i.e. no selective production of either 44 or 46 labelled nitrous oxide which results in random distribution. In all treatments the distribution of  $^{15}\text{N}$  was non-random between nitrous oxide molecules indicating that more than one process was responsible for its production, if only one

process was responsible for nitrous oxide production then there would be a significant difference between the distribution calculations of Stevens and Laughlin (1998). The actual production of  $^{15}\text{N}$  labelled  $\text{N}_2\text{O}$  as a percentage of the total nitrous oxide produced was quantified using the  $m/z$  values for masses 44/45/46, whereby  $\text{N}_2\text{O}$  produced via denitrification was determined using masses 45 and 46 and that via nitrification using mass 44 only, this assumes that nitrification only uses  $\text{NH}_4^+$  and that nitrite produced as a result of nitrate reduction is further reduced to  $\text{N}_2\text{O}$  via denitrification and is not available for nitrification. Figure 44 represents the contribution of nitrification and denitrification to nitrous oxide production following long term soil microcosm incubations and  $^{15}\text{N}$  pool dilution; these data are percentages of fluxes of  $\text{N}_2\text{O}$  as masses 44, 45 and 46; following ANOVA (Minitab, USA) of flux data there were no significant differences ( $p>0.05$ ) between treatments of the nitric and nitrous oxide produced via nitrification and denitrification.

The proportion of total nitrous oxide produced via denitrification following addition of  $^{14}\text{NH}_4^{15}\text{NO}_3$  increased in the microcosms which were amended with acetate and nitrite relative to control and non acetate amendments. This increase of nitrous oxide production via denitrification was greatest in the nitrite amended incubations where 37% of the total  $\text{N}_2\text{O}$  flux was  $^{15}\text{N}$  labelled. In the lime and nitrogen amended incubations there was an increase in nitrification as a source of nitrous oxide relative to control microcosms.

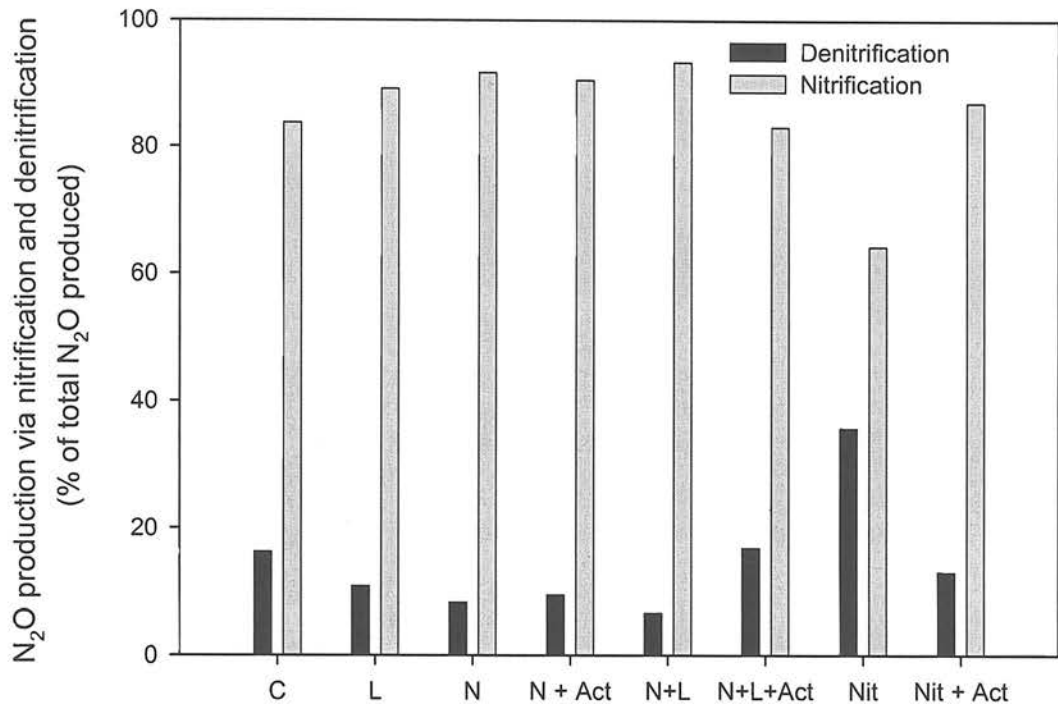


Figure 44. Contribution of nitrification and denitrification to  $N_2O$  production following  $^{15}N$  pool dilution in long term incubation soil microcosms (C – Control; L – Lime; N –  $NH_4NO_3$ ; N + Act –  $NH_4NO_3$  + Acetate; N + L –  $NH_4NO_3$  + Lime; N + L + Act –  $NH_4NO_3$  + Lime + Acetate; Nit –  $NaNO_2$ ; Nit + Act –  $NaNO_2$  + Acetate)

Fractionation factors were determined for both ammonium oxidation to nitrous oxide and nitrate reduction to nitrous oxide following addition of  $^{15}N$  labelled nitrate as ammonium nitrate for all treatment additions. Figures 45 a and 45 b. show the linear regression between  $\log \delta^{15}N$  of the product ( $N_2O$ ) and the associated fractionation factor from nitrate to nitrous oxide and ammonium to nitrous oxide respectively.

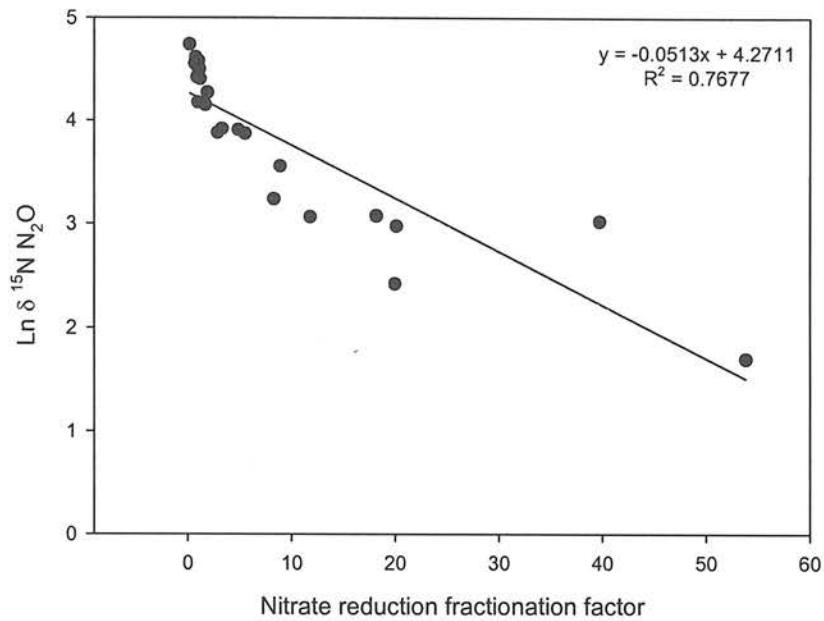


Figure 45a. Linear regression between nitrous oxide and nitrate reduction to nitrous oxide fractionation factors ( $\alpha$ ) for all treatments.

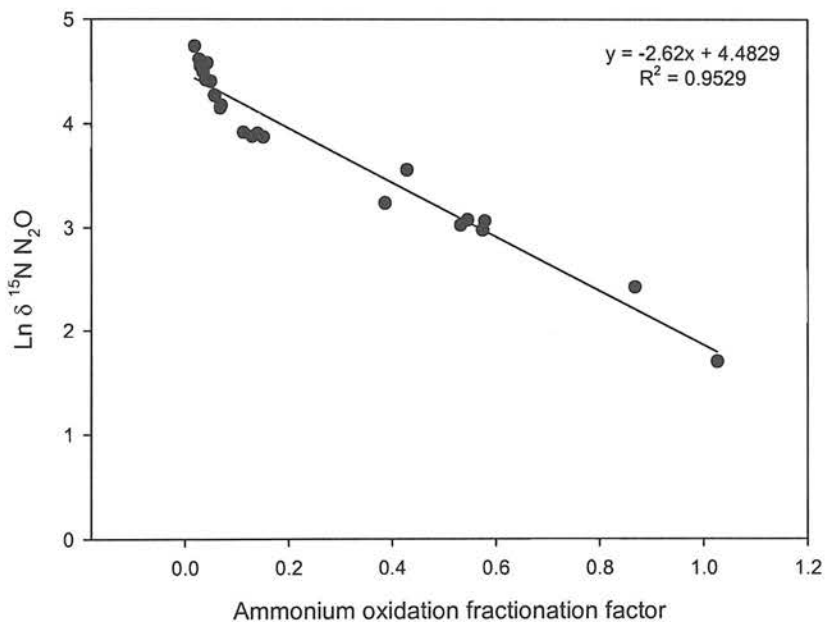


Figure 45b. Linear regression between nitrous oxide and ammonium reduction to nitrous oxide fractionation factors ( $\alpha$ ) for all treatments

The slopes of the linear regression were used in Equation 15 to calculate mean fractionation factors for ammonium oxidation to nitrous oxide and nitrate reduction to nitrous oxide using data for all treatments.

Equation 15. Linear regression equation for fractionation factors of  $N_2O$  formation

$$\delta^{15}N_{Substrate} = \frac{\delta^{15}N_{Product}}{(\alpha - 1) \times 1000}$$

The mean fractionation factors were used to calculate an enrichment factor for nitrous oxide production from either nitrate or ammonium, this indicated the change in product relative to substrate for the production of nitrous oxide. The production of nitrous oxide from nitrate resulted in a much lower enrichment factor of only -0.1‰ compared with that from ammonium to nitrous oxide -3‰, this however is mainly due to the highly enriched  $^{15}N$  of nitrate applied relative to the ammonium which was applied at natural abundance levels.

The flux data for nitric and nitrous oxide were log transformed by adding one to the flux and then taking the  $\text{Log}_{10}$ , a few of the nitrous oxide fluxes were negative hence the addition of one to allow the log to be taken. The results for effects of long term enrichment treatments following pool dilution are shown in Figure 46. There was a significant correlation between log transformed nitric and nitrous oxide except with lime treatments (Figure 46 a) following addition of ammonium nitrate to soil microcosms after 4 months of enrichment. The effects of lime also increased the production of nitric oxide relative to nitrous oxide (Figure 46 a).



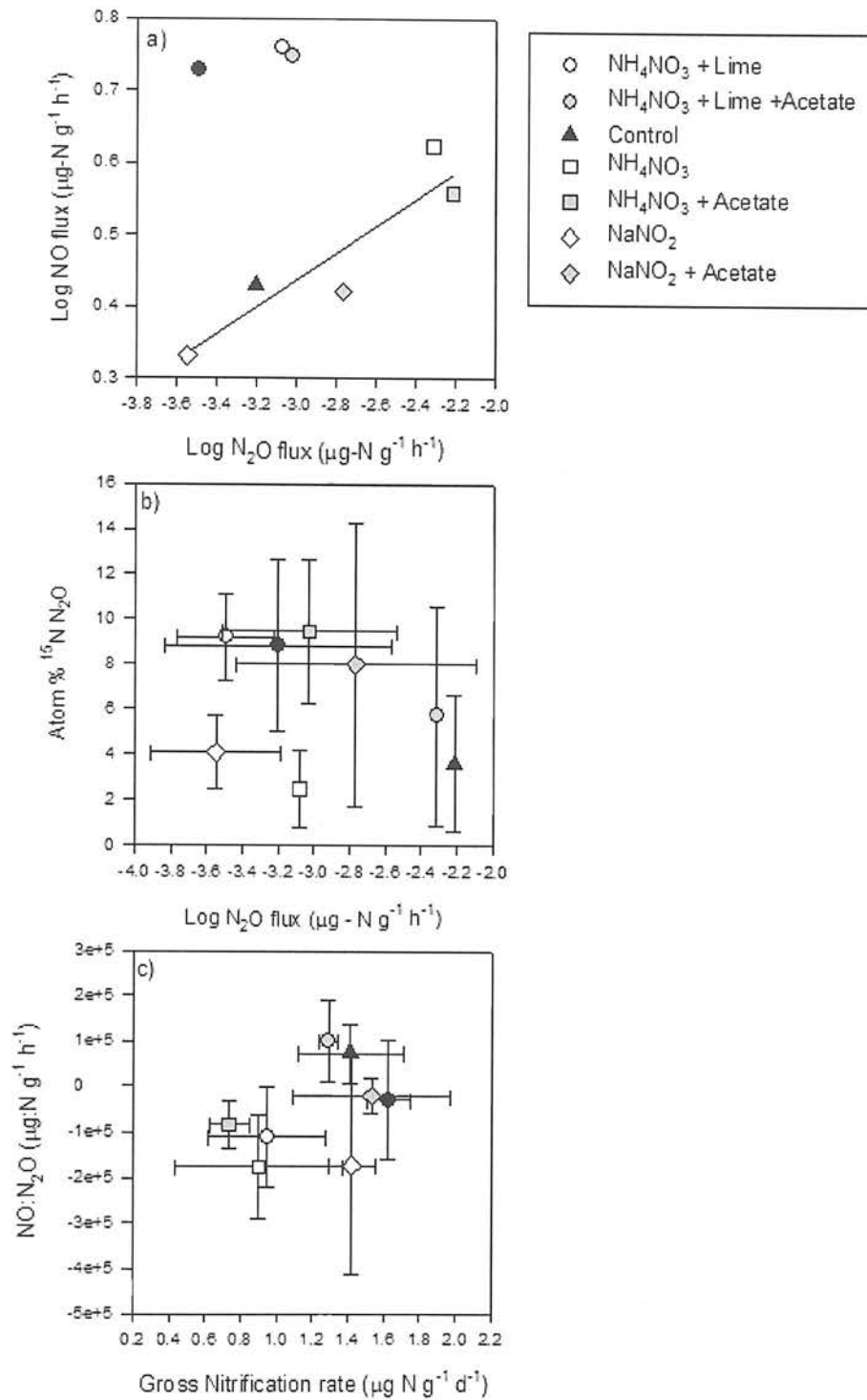


Figure 46. Effects of long term treatment incubations on nitric and nitrous oxide flux (a) (linear regression for data points other than lime treatments,  $R^2 = 1$ ,  $p < 0.001$ ); relationship between nitrous oxide flux and  $^{15}\text{N}$  enrichment of nitrous oxide (b) and gross nitrification rates and nitric to nitrous oxide ratios (c)

The isotopic composition of the nitrous oxide was more enriched in lime treatments which corresponded to lower nitrous oxide fluxes (Figure 46 b). With increasing nitrous oxide flux the  $\delta^{15}\text{N}$  of the nitrous oxide decreased and as nitrification rates increased the nitric to nitrous oxide ratios also increased, Figure 46 c. shows that at lower gross nitrification rates nitrous oxide flux exceeded nitric oxide flux corresponding to nitrogen with and without acetate and nitrite treatments.

#### 6.3.4. Molecular results

Following two months of long term incubation and continuous  $^{13}\text{C}$  enrichment with  $\text{CO}_2$  attempts were made to develop methods for extraction  $^{13}\text{C}$  labelled RNA using ultracentrifugation, up until now these attempts have failed and no heavy bands of RNA have been isolated. Future work will aim to extract  $^{13}\text{C}$  enriched DNA which is less susceptible to degradation as a result of the long centrifugation times required to separate  $^{13}\text{C}$  enriched RNA and DNA on CsCl density gradients.

The results in Figure 47 (a) show differences between populations of heterotrophic eubacteria in the lime treated and nitrogen plus acetate amended microcosms as indicated by the four arrows. Sequence analyses of the four bands indicated in the eubacterial primers were commonly isolated heterotrophic bacteria including *Stenotrophomonas* and *Acinetobacter*.

The effects of incubation treatments on the  $\beta$  ammonium oxidisers shown in Figure 47 (b) indicate *Nitrospira* AF bands in the nitrite treatment only with no changes in the  $\beta$  ammonium oxidisers for other treatments.

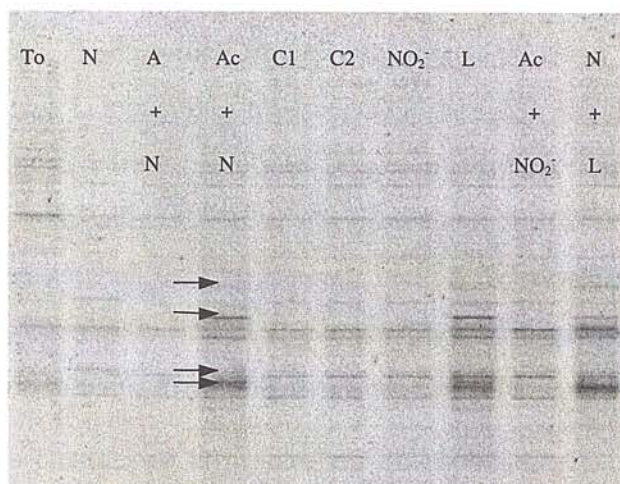


Figure 47 a. DGGE analysis of 16S rRNA genes amplified from extracted DNA using eubacteria, primers. C – control, N –  $\text{NH}_4\text{NO}_3$ , L – lime, Ac – acetate,  $T_0$  – initial value,  $\text{NO}_2^-$  –  $\text{NaNO}_2$ . Arrows indicate changes in eubacterial genes for N+Ac; L and N + L treatments. (Courtesy of Prof. J. Prosser, Univ. of Aberdeen).

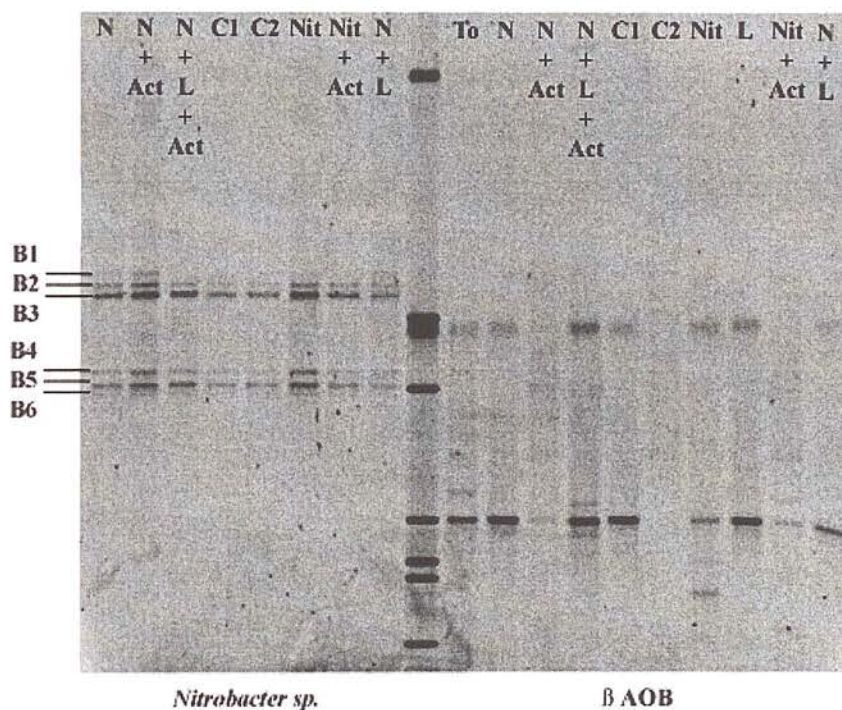


Figure 47 b. DGGE analysis of 16S rRNA genes amplified from extracted DNA using *Nitrobacter* and AOB primers. C – control, N –  $\text{NH}_4\text{NO}_3$ , L – lime, Act – acetate,  $T_0$  – initial value, Nit –  $\text{NaNO}_2$ . B1 to B6 correspond to 6 different *Nitrobacter* sp. (Courtesy of Prof. J Prosser, Univ. of Aberdeen)

Following phylogenetic analyses of the *Nitrobacter* type sequences derived from the microcosm incubations (Figure 47 b) all but one of the sequences were found to be similar to current collections of pure cultures of *Nitrobacter* sp.. The *Nitrobacter* sp. ATCC25385 which was closely related to Band 5 isolated from the N, N+L and Ac + N + L incubation treatments (Figure 48) was the only identifiable sequence. Bands 2 and 4 were closely related to *Bradyrhizobium japonicum* which forms part of the alpha- subdivision of the proteobacteria (Teske *et al.*, 1994) and positively correlated with all lime treatments, band 6 from columns were positively correlated with nitrogen plus lime and acetate plus nitrogen plus lime treatments which were closely related to *Bradyrhizobium* sp. ORS278.

The relative changes in the microbial functional groups as a result of long term incubation compared with control soil DGGE banding profiles are shown in Figure 48. Addition of nitrogen increased the number of DGGE bands in the AOB and *Nitrobacter* sp. profiles. Changes in the eubacterial DGGE bands were mediated by lime + nitrogen + lime and nitrogen + lime + acetate treatments in which the number of bands increased relative to control incubations. Land management treatments used in this long term incubation experiment either increased or had no affect on the number of DGGE bands relative to control soil incubations, nitrogen + acetate (Figure 49.) however, did result in a decrease in the number of eubacterial DGGE bands relative to the control treatments.

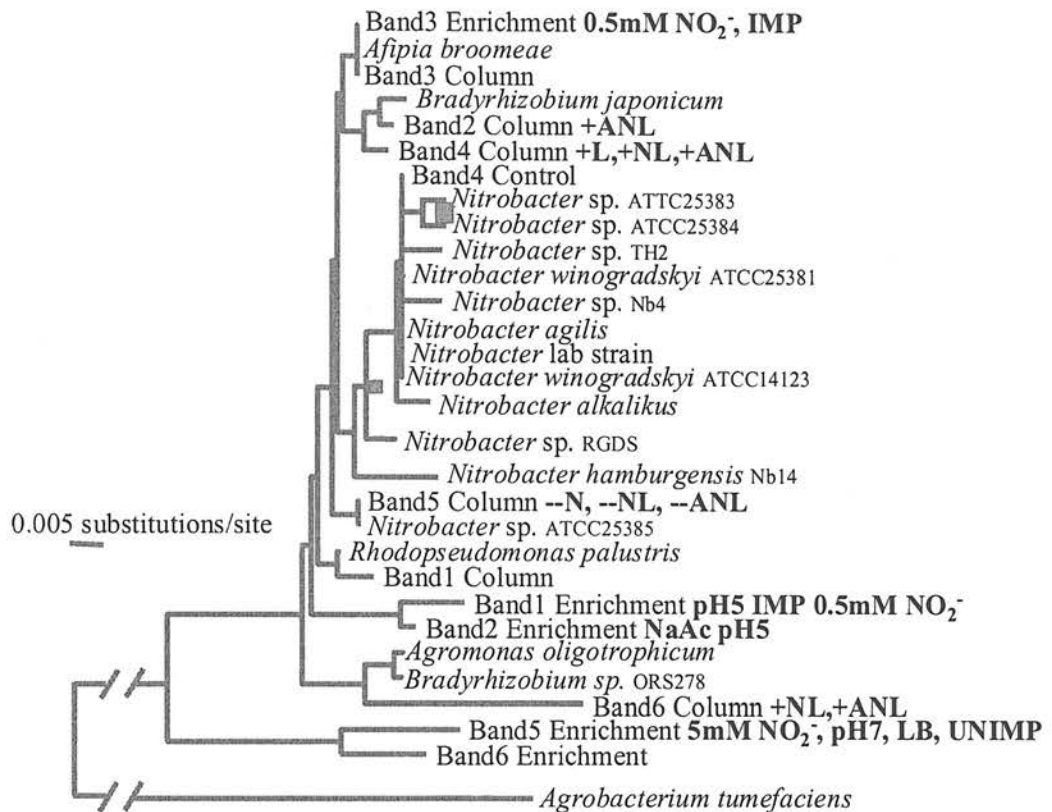


Figure 48. Phylogenetic tree for *Nitrobacter* like species isolated from soil incubations, + and - denote positive and negative correlations with the following treatments C - Control; L - Lime; N - NH<sub>4</sub>NO<sub>3</sub>; N + Act - NH<sub>4</sub>NO<sub>3</sub> + Acetate; N + L - NH<sub>4</sub>NO<sub>3</sub> + Lime; N + L + Act - NH<sub>4</sub>NO<sub>3</sub> + Lime + Acetate; Nit - NaNO<sub>2</sub>; Nit + Act - NaNO<sub>2</sub> + Acetate. Additional treatments refer to enrichment cultures isolated from Sourhope soil. (Figure courtesy of Prof. J. Prosser, Univ. of Aberdeen)

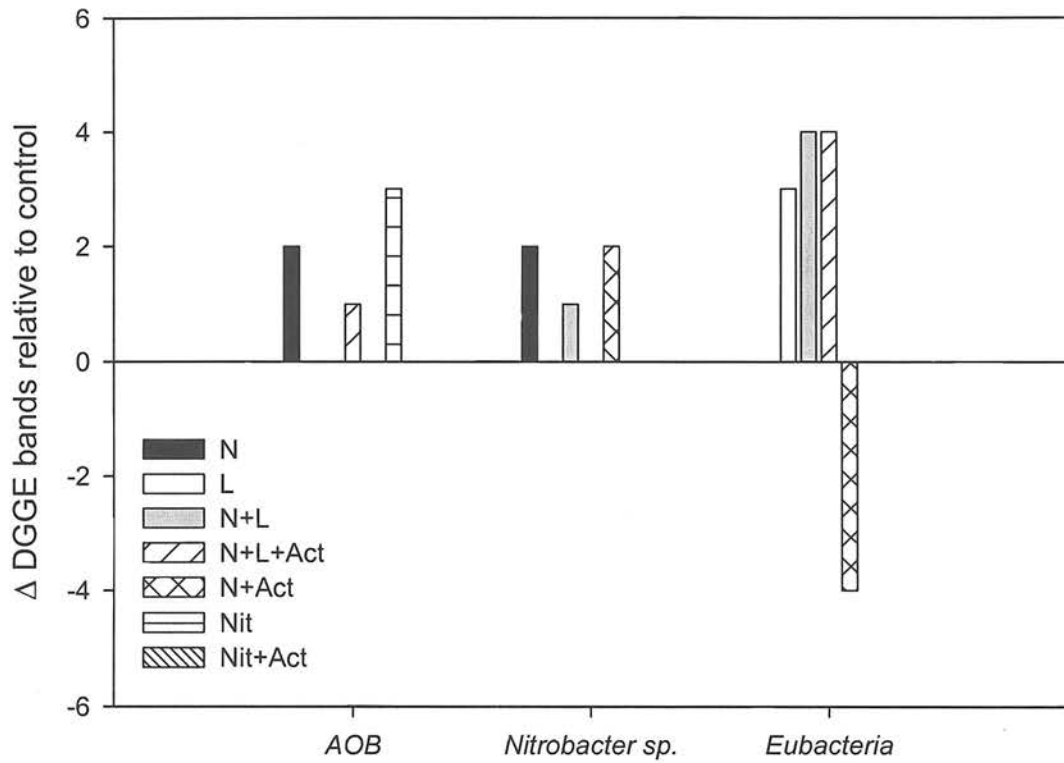


Figure 49. Changes in the DGGE banding profiles for  $\beta$  AOB, *Nitrobacter* sp. And Eubacterial primers relative to control soil banding profiles. (C – control; L – Lime ( $\text{CaCO}_3$ ); N –  $\text{NH}_4\text{NO}_3$ ; N + Act –  $\text{NH}_4\text{NO}_3$  + Acetate; N + L –  $\text{NH}_4\text{NO}_3$  + Lime; N + L + Act –  $\text{NH}_4\text{NO}_3$  + Lime + Acetate; Nit –  $\text{NaNO}_2$ ; Nit + Act –  $\text{NaNO}_2$  + Acetate)



## 6.4. Discussion

This study showed that land management regimes in upland agricultural grassland soils may result in increased N loss as nitric and nitrous oxide via both nitrification and denitrification at 60% WFPS. Increases in both nitric and nitrous oxide emissions could not be correlated with increases in gross nitrification rates due to the large variation within treatments, however the proportion of nitrous oxide produced via nitrification was greater than that produced via denitrification for all treatments.

There were shifts in the number of bands for all three functional groups measured using DGGE 16sRNA, although changes in the eubacterial DGGE profiles were greater indicating that both the AMO and *Nitrobacter sp.* ammonium and nitrite oxidisers were relatively stable.

The treatment effects on the changes in the specific microbial functional groups as determined by the DGGE banding patterns showed that lime, alone or in combination with nitrogen or nitrogen plus acetate increased the number of eubacterial microbial species relative to non amended control treatments. The changes in eubacterial DGGE banding profiles in the lime treatments may be associated with changes in function; where the nitric to nitrous oxide ratio was greater in the lime treatments than non limed treatments (Figure 47 a) which is associated with increased nitrification rates (Šimek and Cooper, 2002).

The two most common eubacteria identified from the DGGE banding profiles were *Stenotrophomonas* and *Acinetobacter*. The *Stenotrophomonas* species form part of the gamma sub class of the *Proteobacteria* and studies have shown that they are capable of nitrite and nitrate reduction with end product of nitrous oxide (Wolfgang

*et al.*, 2000). The percentage of nitrous oxide produced via denitrification in the lime treatments however was less than that produced in the control treatments, although the  $^{15}\text{N}$  enrichment of nitrous oxide produced was greater than or equal to that of the control treatment in the lime and nitrogen + lime + acetate treatments. Despite the low proportion of the total nitrous oxide emission from denitrification in the lime treatments the production of  $^{15}\text{N}$  labelled nitrous oxide may be associated with nitrate reduction by the *Stenotrophomona* species as indicated by increased DGGE banding in the lime treatments.

The addition of lime also had an effect on the flux of both nitric and nitrous oxide, increasing the flux of nitric oxide relative to that of nitrous oxide. Soil pH has been shown to significantly affect distribution patterns of autotrophic nitrifier strains isolated from agricultural soils (Koops and Pommerening-Roser, 2001). A review of nitrification in acid soils by De Boer and Kowalchuk (2001) indicated that autotrophic rather than heterotrophic nitrification was the dominant process in terms of nitrogen cycling in most acid soils, although the contribution by heterotrophic nitrification is difficult to assess due to their functional diversity for example the nitrifying denitrifiers (Robertson and Kuenen, 1991). The addition of lime increased the nitric oxide flux relative to nitrous oxide flux when compared with all other treatments, this change in function may also be linked with changes in molecular DGGE banding profiles of the eubacteria in lime treatments, therefore nitrification at Sourhope could be predominantly heterotrophic. This is only true if there is a direct cause and effect of changes in molecular diversity upon function measured as a change in the nitric and nitrous oxide flux.

It must be stressed that these links between function and diversity are merely speculative and direct links may only be quantified with  $^{13}\text{C}$  labelling of RNA and DNA (Boschker *et al.*, 1998; Bull *et al.*, 2000), although tentative links have been found between soil improvement and bacterial community structure by other research groups involved with the NERC Soil Biodiversity programme at Sourhope. Gray *et al.* (2003) quantified autotrophic ammonium oxidation and microbial community structure in the lime treatments in the field at Sourhope, they measured significant differences in potential ammonium oxidation between lime amended and

un-improved control soils with increased rates in the lime treated soils. The data in this study also showed increases in nitrification rates and nitric and nitrous oxide emissions in the lime amended soils, Gray *et al.* (2003) however, used TGGE (temperature gradient gel electrophoresis) to quantify eubacterial and AOB community composition they noted clustering of eubacterial TGGE profiles in the lime treatments and found that this treatment had the greatest effect on the bacterial community composition.

Both the AOB and *Nitrobacter sp.* increased in terms of DGGE banding patterns compared with control treatments, again bands were positively correlated with nitrogen and lime amended soils. Recent advances in the use of quantitative real time PCR of ammonia oxidising bacteria in soils have shown that with increased addition of ammonia the number of ammonia oxidisers also increased (Okano *et al.*, 2004), the differences between the emission of nitric and nitrous oxide in the one week and 6 month application rates for N additions are probably in part due to increased numbers of ammonium oxidisers, although Okano *et al.* (2004) also noted slight increases in the overall total bacterial population size some of which may also be capable of nitric and nitrous oxide production.

The *Nitrobacter* sequences identified from DGGE banding profiles were closely related to *Bradyrhizobium sp.* (Figure 47) and correlated with the lime, nitrogen + lime and nitrogen + lime + acetate treatments corresponding to bands 2, 4 and 6. Bands 2 and 4 were most closely related to *Bradyrhizobium japonicum* which posses several if not all the denitrification enzymes and are capable of both nitrous oxide and N<sub>2</sub> production from the reduction of nitrate under anaerobic conditions (Sameshima-Saito *et al.*, 2004), whereas band 6 was most closely related to *Bradyrhizobium sp.* ORS278.

Interactions between plant species diversity and below ground microbial processes may also play an important role in driving below ground function in upland grassland soils (Grayston *et al.*, 2004). Dawson *et al.* (2004) quantified root turnover rates and carbon and nitrogen input to soil at the Sourhope Soil Biodiversity field site, in the nitrogen plus lime amended plots they noted increased N and C input

to the soil rhizosphere relative to control plots with corresponding increases in the number of bacteria and diversity of microorganisms. The findings of Dawson *et al.* (2004) complement those of this study, nitrogen in combination with lime resulted in increased NO:N<sub>2</sub>O and associated changes in the eubacterial and *Nitrobacter sp.* DGGE banding profiles, however in these experiments above ground biomass was removed and therefore treatment effects may be enhanced in the field with supplementary C and N input from the rhizosphere as a result of increased root turnover (Dawson *et al.*, 2004; King *et al.*, 2002).

The addition of acetate increased the nitric and nitrous oxide emissions relative to non acetate amended treatments at the low application rates, but not at the 21 week application rates. The increased emission of both nitric and nitrous oxide with acetate additions in these experiments could be due to increases in heterotrophic nitrification. Organic carbon is important for heterotrophic microbial processes, heterotrophic nitrification and oxidation of ammonium coupled to the reduction of organic carbon by the denitrifier *Thiosphaera pantotropha* was shown to occur under aerobic conditions in the presence of an organic electron donor (acetate) (Robertson and Kuenen, 1991), this nitrite produced may then be further reduced to other intermediates of the denitrification pathway and result in nitric or nitrous oxide emission.

The production of <sup>15</sup>N labelled nitrous oxide in the acetate amended treatments following the <sup>15</sup>N pool dilution indicated that nitrification was the predominant source of nitrous oxide, gross nitrification rates were also greater in the nitrogen + acetate and nitrite + acetate treatments relative to non acetate analogues.

It is also known that in glucose amended soils denitrification rates may be increased (Zumft, 1997), for example Lind and Eiland (1989) detected significant increases in nitrous oxide production in a sandy-clay soil amended with organic carbon in the form of glucose, the production was further enhanced under anaerobic conditions and therefore attributable to denitrification. Addition of organic carbon in the form of acetate did increase nitrous oxide emissions in Sourhope soil (Figure 40), however the analysis of the <sup>15</sup>N enrichment of nitrous oxide produced indicated that with the acetate amended nitrogen treatments the source of nitrous oxide was

predominantly via ammonium and not nitrate. The addition of acetate to nitrogen and lime and nitrite treatments resulted in enrichment of nitrous oxide  $^{15}\text{N}$  similar to that of the  $^{15}\text{N}$  of nitrate applied, and could therefore be attributed to denitrification or dissimilatory nitrate reduction to ammonium (DNRA), the latter of which is unlikely since the  $^{15}\text{N}$  of ammonium was not enriched (Figure 43).

The use of stable isotope ratios for discriminating between sources of nitrous oxide has been studied extensively at natural abundance levels (Bergsma *et al.*, 2001; Stevens *et al.*, 1998; Stevens and Laughlin, 1998; Bergsma *et al.*, 1999). The application of these methods at enriched levels was used to elucidate fractionation factors and potential sources of nitrous oxide. The nitrogen, nitrogen plus acetate, nitrogen plus lime and nitrite treatments had low atom%  $^{15}\text{N}$   $\text{N}_2\text{O}$  similar to that of the ammonium atom %  $^{15}\text{N}$ , following further analyses of the  $^{15}\text{N}_2\text{O}$  data the proportion of nitrous oxide produced via nitrification and denitrification was quantified, although there were no significant differences between treatments, nitrification as a source of nitrous oxide was greater in lime and nitrogen amended soil microcosms.

Elevated nitrification rates were found in the control; lime; nitrite plus acetate and nitrogen plus lime plus acetate treatments, which also had high  $\delta$   $^{15}\text{N}$   $\text{N}_2\text{O}$  enrichment similar to that of the labelled nitrate.

Incubation of the Sourhope soil with land management treatments did result in changes in function and also subsequent changes in molecular diversity, this was emphasised in the nitrite and lime treatments in terms of molecular diversity. These changes were mainly mediated via an effect on heterotrophic molecular diversity and function in terms of nitric and nitrous oxide production. Nitric and nitrous oxide production via nitrite was attributed to abiological reactions (discussed in Chapter 2).

The addition of lime during the course of the experiment did not affect measured soil pH, although dissolution of the  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  ions which could have an immediate effect on the pH of the soil water within the soil pore space at the microbe/nutrient/soil interface where active populations of bacteria are present (Borken and Brumme, 1997).

The predominant treatment effects of lime on both function and microbial community composition indicate that changes in nitrification could be due to changes in the eubacterial heterotrophic populations and land management treatments could change this system from a predominantly heterotrophic to an autotrophic system in terms of nitrification.

This has implications for ecosystem resilience and gaseous nitrogen losses; autotrophs tend to grow much more slowly and require inorganic nitrogen either from mineralisation or N input for energy and are less able to compete for N than heterotrophs or plants. Heterotrophs have a low affinity for nitrogen but high rates of N cycling. Autotrophs are also highly specialised although both nitrifiers and methane oxidisers are able to oxidise both ammonium and methane via AMO and MMO enzymes respectively (Hanson and Hanson, 1988), whereas heterotrophs are able to switch function, which is desirable in terms of ecosystem function resulting in enhanced resilience to a stress either nutrient or environmental.

The nitrogen input can be optimised so that N cycling is at its most efficient for plant productivity and least gaseous N loss and leaching rather than from a loss of species point of view. It is reasonable to assume that microorganisms are like air filters, and regulate greenhouse gases to produce stable ecosystems, it is when these greenhouse gases are supplemented by anthropogenic inputs that the system will be most stressed and the ability to switch function becomes most important. The autotrophs could be the fine scale whereas the heterotrophs are the course scale regulators of greenhouse gases in the atmosphere. Land management needs to not only increase crop yields but also allow for natural supplementation of N via the nitrogen cycle and maintain both autotrophic and heterotrophic functional diversity.

However with more and more research pointing to the fact that soil microorganisms tend to be there all of the time in terms of molecular diversity, perhaps it is only from a functional point of view that land management decisions need to be made.

Putative links between changes in the function and species composition of functional groups within the nitrogen cycle may be influenced by land management



regimes in upland agricultural grassland soils. Addition of lime nitrogen and a readily available organic carbon source resulted in increased gross nitrification rates associated increased nitric and nitrous oxide emissions, these changes in function coincided with simultaneous changes in the eubacterial and *Nitrobacter sp.* communities. These data therefore indicate that land management regimes may cause increases in greenhouse gas emissions associated with changes in microbial functional groups, *Nitrobacter sp.* and eubacterial DGGE banding profiles may be potential indicators for quantifying potential losses of nitric and nitrous oxide due to N additions in upland grasslands.

## Chapter Seven. Field flux measurements of nitric and nitrous oxide.

### 7.1. Introduction

The Natural Environment Research Council (NERC) Soil Biodiversity thematic programme was established in 1997, with an aim to fully integrate a field scale study of an upland grassland ecosystem.

The field site for the NERC Soil Biodiversity programme lies 15 miles south of Kelso at the head of the Bowmont valley, on the western slopes of the Cheviots (Scottish uplands). The site is an upland grassland system, which is based at Macaulay Land Use Research Institute Sourhope Research Station, and is part of the UK Environmental Change Network.

The earliest records of the Sourhope field site as a farm date back to the 14th century and the name is said literally to mean 'the valley of sour pastures'.

Work done by Davidson *et al.* (1999) at the Sourhope field site as part of the Soil Biodiversity programme have noted that there was cultivation in the rig and furrow, manuring and addition of charcoal until the early 18<sup>th</sup> century. There was also evidence of homogenisation of the Ap horizon only. When cultivation was abandoned the soil developed a surface horizon LF and H and the introduction of thickness variation in relation to drainage differences (Davidson *et al.*, 1999).

The soil Biodiversity programme was set up exclusively to quantify the effects of land management as fertilizer and lime addition on all aspect of soil biological diversity in a previously un-managed upland Grassland in the Scottish borders. The field site is situated latitude 2°15W and longitude 55°30N and 320m above sea level. The soil is from the Sourhope series with an annual rainfall of 2.9 mm

The chemical properties of soils can be altered as a result of liming and nitrogen additions. Šimek *et al.* (1999) showed that soil C and N contents increased with addition of inorganic fertiliser N and that the addition of lime did not change the soil pH, the addition of fertiliser N alone on the other hand resulted in a decrease in

the soil pH associated with increases in nitrifying bacteria and production of nitrite. The effects of nitrogen addition in grasslands results in losses of both nitric and nitrous oxide which normally account for less than 2% of the total N applied as fertiliser (inorganic) (Vermoesen *et al.*, 1996) and 1.25% of N input is emitted as N<sub>2</sub>O (IPCC, 2001).

Ryden (1981) noted that application rates of ammonium nitrate at 250 kg N ha<sup>-1</sup> yr<sup>-1</sup> resulted in both consumption and production of N<sub>2</sub>O, conditions under which the soil acted as a net sink for nitrous oxide were under dry soil conditions combined with low nitrate concentrations. The nitrous oxide uptake can be mediated via nitrous oxide respiration by denitrifiers when nitrate becomes limiting (Zumft, 1997). Vermoesen *et al.* (1996) looked at the effects of fertiliser N additions (0, 50, 100 and 150 mg N kg<sup>-1</sup> soil) and found that fertiliser addition had a significant effect on nitric oxide production, and that the rate of nitrous oxide production was totally dependant on soil characteristics with a linear increase in production with increasing inorganic N concentration.

Liming has been shown to increase rates of nitrogen mineralisation in grassland soils (Whitehead, 1995). With liming of soil from pH X to pH 6.7 there was a doubling of the soil N mineralised (Nyborg and Hoyt, 1978), thus providing increased concentrations of inorganic nitrogen for loss of N as nitric and nitrous oxide via nitrification and denitrification. The effects of lime and nitrogen not only affect emission of nitric and nitrous oxide but also the mole fraction of N<sub>2</sub>O and N<sub>2</sub> released (Haynes, 1986). This mole fraction was found to increase as the pH of soils decreased. Smith and Chalk (1980) noted that immobilisation of nitrite and associated gaseous nitric and nitrous oxide evolution was inversely related to soil pH.

In soils where denitrification is the dominant source of nitrogenous gases then N<sub>2</sub>O emissions tend to decrease with increasing pH in acid soils (pH below 5-6) (Granli and Bockman, 1994) although the rate of denitrification has been shown to increase with increasing pH (optimum pH 7 – 8) (Conrad, 1995), this increase in rate and associated decrease in N<sub>2</sub>O production is due to a decrease in the N<sub>2</sub>O:N<sub>2</sub> product ratio of denitrification (Haynes, 1986).

The production of nitric oxide from acidic soils is sometimes greater than production from less acidic soils (Conrad *et al.*, 1996). The microbial activity in soils is determined primarily by the soil carbon content as this provides both a source of energy and cellular carbon (Conrad, 1995). Studies of denitrification in permanent pasture soils showed that the availability of organic carbon in the form of glucose reduced the  $\text{N}_2\text{O}:\text{N}_2$  ratio as a result of increased nitrous oxide reduction via denitrification (Dendooven *et al.*, 1996).

Previous studies have noted marked diurnal patterns in NO fluxes as a result of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  additions to soil (Bisson, 1994), the diurnal flux at Sourhope could contribute a large proportion of the annual flux via denitrification due to observed increases in root exudates during the night (DOC) providing substrate for heterotrophic denitrification (Ineson, pers. Comm.).

This study aimed to quantify the effects of lime and nitrogen application to an unimproved upland agricultural grassland soil on the emission of nitric and nitrous oxide over a 2 year period.

The hypothesis for this study was that application of nitrogen and lime alone and in combination would result in net emission of nitric and nitrous oxide mediated by both nitrification and denitrification under low and high soil moisture contents respectively. It was therefore also hypothesised that timing of fertiliser application and emission of nitric and or nitrous oxide would be closely linked to soil environmental conditions, following nitrogen application under dry soil conditions nitric oxide would be the dominant gas emitted whereas nitrous oxide would be greater under high soil moisture contents following fertiliser application.

## 7.2. Materials and Methods

### 7.2.1. Soil and vegetation

The field site is 320 m above sea level and 55°28'N/2°14'W, the soil forms part of the Sourhope series and the major sub-group is that of a brown forest soil (%N 0.56, %C 7.61, pH (H<sub>2</sub>O) 4.54-4.81, pH (CaCl<sub>2</sub>) 4.04-3.88).

The vegetation at the site is predominantly that of the National Vegetation Classification (NVC) community U4d: *Festuca ovina* L. (native perennial which forms thick tufts and is common on poor soils particularly on hills or moorland) – *Agrostis capillaris* – *Galium saxatile* (mat-forming perennial herb) grassland, *Luzula multiflora* (Retz.) Lej. (native perennial, common on acid or peaty soil) – *Rhytidiadelphus loreus* (Hedw.) Warnst. (Common moss found on acid soil) sub-community.

The field site was arranged around a random block design, each block was further divided into sub-plots with further sub-treatments made in addition to the main block treatments. The main block treatment additions at the Sourhope field site are intended to reflect those, which are used in upland agricultural systems. For detailed site description and treatment structure see Chapter 2.

### 7.2.2. Gaseous N measurements

Nitric oxide measurements were made using a Thermoquest 42 CTL NO<sub>x</sub> trace analyzer and static chamber techniques. For detailed description of field flux measurements see Chapter 2. Briefly, chamber lids were placed on the chambers in each block at 2-minute intervals; these were then capped for 15 minutes before analyzing the outlet sample tube using the NO<sub>x</sub> analyzer. The lid was collapsed to prevent pressure effects from the high flow rate of the analyzer during the 2 minute sample period. Data was logged using a Campbell 21X data logger (Campbell Scientific, USA) connected to a laptop computer.

Nitric oxide measurements were made on the following dates 05/07/00; 13/06/00; 17/06/01; 24/07/01 and were restricted to dry weather conditions due to the requirement for mains power for the NO<sub>x</sub> analyzer.

Nitrous oxide measurements were made using static chamber techniques; an ambient air sample was drawn using a 1000ml syringe (Hamilton) prior to capping of each block of chambers. Chambers were then capped for 60 minutes before sampling of 500 ml of gas into Tedlar bags for analyses back at the lab. The GC system consisted of a Chrompak CP 9000 with a Poropak Q column with the oven temperature set to 30°C and the ECD detector and injection port to 350°C.

Measurements of nitrous oxide were made on the following dates 18/05/00; 13/06/00; 05/07/00; 13/10/00; 03/11/00; 22/05/01; 30/05/01; 22/05/01; 30/05/01; 05/06/01; 21/09/01; 22/11/01.

### **7.2.3. Soil sampling and inorganic N**

Soil was sampled from around the static chambers of each block replicate using a screw auger. The screw auger sampled to a depth of 10 cm and diameter of approximately 4 cm, giving a soil sample of 40g wet weight. Due to the impacts of foot and mouth disease on the Scottish Borders soils samples were taken by MLURI and stored at 5°C until it was possible to retrieve samples from the field site following the end of the foot and mouth disease crisis.

Samples were analysed using the Kontron continuous flow system colorimetrically following extraction with 1M KCl and filtration using Whatman 42 filter papers as described in chapter 2.

### **7.2.4. Diurnal field flux measurements**

Diurnal field measurements were restricted to block three due to the health and safety considerations with moving the NO analyzing equipment around the field site in the dark. The analyzer was fitted with a 30m PTFE sample tube inlet; this was tested to make sure that there were no reactions with ozone affecting the NO due to



the long sample line. The tests on the sample line were performed by using an NO gas calibration system (Thermoquest). A known NO concentration was measured using the NOx analyzer, any reactions with ozone would result in a significant difference between the NO measured and the NO concentration from the calibration system.

Measurements of both nitric and nitrous oxide were made using the static chamber techniques described in Chapter 2 every two hours throughout a 24 hour period.

## 7.3. Results

### 7.3.1. Soil moisture and temperature

Daily mean soil temperature, moisture, and rainfall measurements are shown in Figure 50 b, c and d for the periods covering the soil inorganic N and gas flux measurements. Maximum soil temperatures were 14°C at 0-5cm depth which occurred during the warmer summer months of May through to July and August with minimum temperatures of 1°C in December when the soil was often frozen.

The soil moisture contents determined using the automatic weather station (AWS) were lower in summer when the soil temperatures were high and greatest in winter when rainfall was high and soil temperatures low (Figure 50 b – d).

Soil WFPS measurements were made following analysis of inorganic N in the field and these correlated with periods of high rainfall and high soil moisture content and also period of high soil temperatures with lower rainfall, particularly in June 2001 where soil WFPS decreased significantly (ANOVA,  $p < 0.05$ ) from a mean moisture content of 80% WFPS on the 30/05/01 to between 40 and 50% WFPS on the 05/06/01. This corresponded to a period of very little rainfall, with an average of 0.7 mm between the two sample dates and warm hot sunny days with a mean radiation of 19.6 MJoules m<sup>-2</sup> and soil temperature of 12°C. The average soil moisture content at the field site was 50% WFPS with mean soil temperatures of 8.5°C and rainfall of 2.9 mm during the field measurement campaign.

Very high rainfall was measured on the 06/11/00 to 08/11/00 with a total rainfall of 193 mm over the three days with soil moisture contents reaching  $0.5 \text{ m}^3 \text{ m}^{-3}$ , almost double the yearly average.

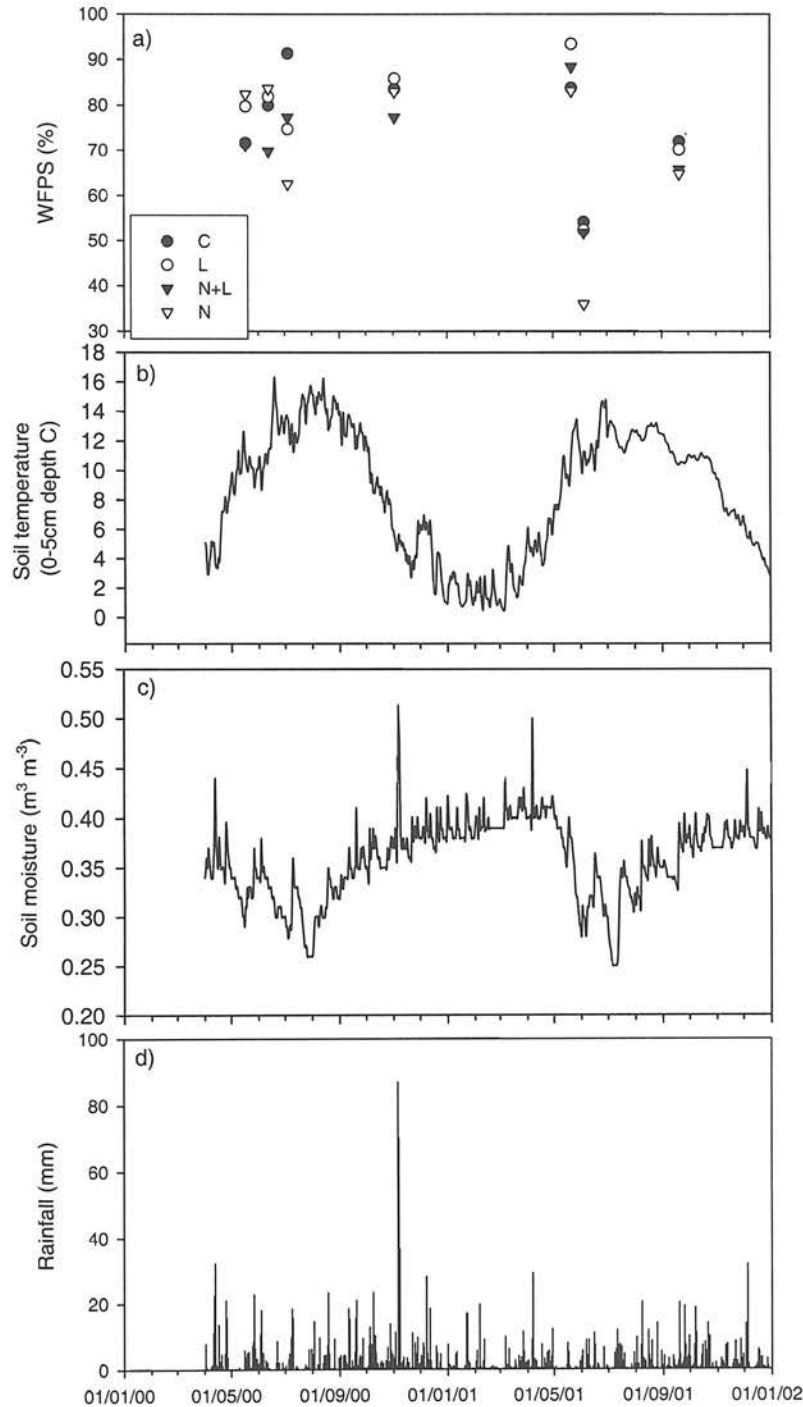


Figure 50. Sourhope field soil temperature (b); moisture content (c) and rainfall (d) daily means logged using an AWS from April 2000 until November 2001. Soil measurements of WFPS (a) were made during inorganic N sampling (N =  $\text{NH}_4\text{NO}_3$ , L =  $\text{CaCO}_3$ , C = control, N+L =  $\text{NH}_4\text{NO}_3 + \text{CaCO}_3$ ).

### 7.3.2. Nitric and nitrous oxide flux

The addition of nitrogen at 126 kg N ha<sup>-1</sup> yr<sup>-1</sup> resulted in significant (ANOVA,  $p < 0.001$ ) increases in the flux of both nitric and nitrous oxide (Figure 51 a and b) in both the nitrogen and nitrogen + lime plots. Following analyses of variance with block in the general linear model, there were no significant effects of treatment block on the emission of nitric and nitrous oxide indicating that the spatial variability at Sourhope does not affect emissions measured at these scales.

The emissions of nitric oxide in contrast to nitrous oxide were greatest following the field treatment application in 2001, with nitrogen + lime and nitrogen treatments resulting in largest net emission from the soil. The largest emission of nitrous oxide was measured during the 2000 field season again immediately after treatment applications were made.

The nitrous oxide flux measurements made on the 18/05/00, 13/06/00 and 05/07/00, two weeks after the second treatment addition; were significantly greater in nitrogen and nitrogen plus lime treatments compared with control and lime treatments (ANOVA,  $p < 0.01$ ). The emission of nitrous oxide following treatment applications in 2000 however, significantly decreased on the 05/07/00 to 0.01  $\mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$  and then became a net sink (ANOVA,  $p < 0.0001$ ).

The flux of nitric oxide did not increase following the initial treatment applications in the field with a very low flux of 0.26  $\mu\text{g NO} - \text{N m}^{-2} \text{ h}^{-1}$  in the nitrogen treated plots. The nitric oxide flux however; did increase following application of nitrogen in 2001. The corresponding increase in nitrous oxide flux in the nitrogen and nitrogen + lime treatments was not significant. The flux of nitric oxide was greatest in the nitrogen and lime treatments (Figure 51 b) with a maximum flux of 4.57  $\mu\text{g NO} - \text{N m}^{-2} \text{ h}^{-1}$  on the 24/07/01 almost 2 months after treatment application.

The flux of nitric oxide on a N for N basis was much greater than that of nitrous oxide even when comparing periods of maximum flux for both gases. Nitrous oxide fluxes increased quite rapidly following N additions in nitrogen and nitrogen

plus lime treatments and both decreased with time, this corresponded to high soil moisture contents (Figure 50 a).

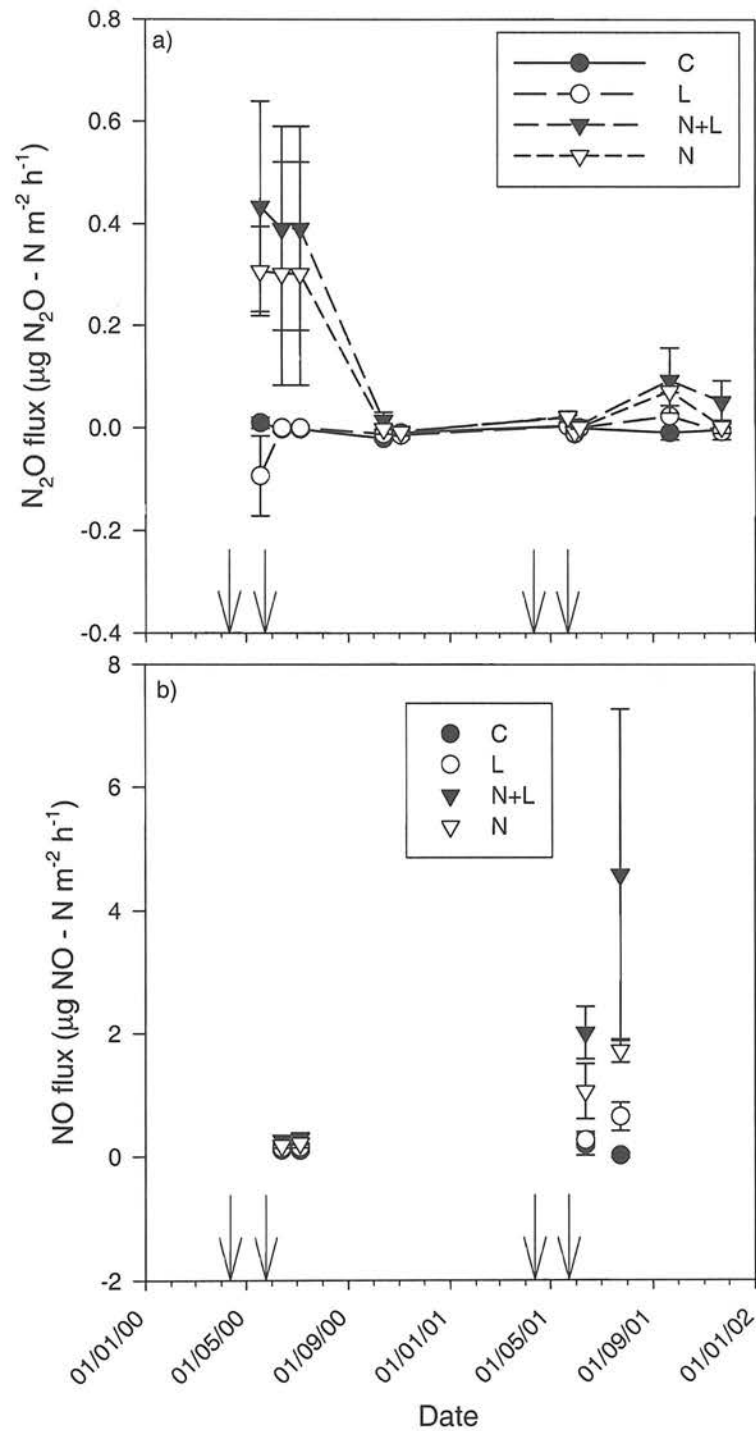


Figure 51. Effects of land management on nitric and nitrous oxide flux in the field. Arrows indicate treatment application dates, C = control, L = CaCO<sub>3</sub>, N = NH<sub>4</sub>NO<sub>3</sub>, N+L = NH<sub>4</sub>NO<sub>3</sub> + CaCO<sub>3</sub> (Error bars = ± S.E., n=3)

Both fluxes of nitric and nitrous oxide were greatest in nitrogen plus lime and nitrogen only treatments followed by lime and then control treatment applications (Figures 51 a and b). Increased nitric and nitrous oxide flux also correlated with soil inorganic N, corresponding to treatment applications.

### 7.3.3. Soil inorganic nitrogen

Changes in the soil exchangeable ammonium and nitrate pools are shown in Figures 52 a and b. Both nitrate and ammonium pools increased following addition of ammonium nitrate in the field in the nitrogen and nitrogen plus lime treatments. The soil exchangeable ammonium pool increased from  $4.7 \mu\text{g N g dry weight soil}^{-1}$  in the control plots to  $13 \mu\text{g N g dry weight soil}^{-1}$  in nitrogen treated plots following treatment additions in the field.

The nitrate and ammonium soil inorganic N increased following the second N application, although treatment effects were not significant. Unusually high ammonium concentrations were measured in control plots on the 22/11/01 similar to those of the nitrogen amended plots  $123 \mu\text{g N g dry weight soil}^{-1}$  and  $73 \text{ mg N g dry weight soil}^{-1}$  respectively. These were following a period of low rainfall with a soil moisture content of 65% WFPS (Figure 50 a).

Generally both soil nitrate and ammonium concentrations significantly (ANOVA,  $p < 0.0001$ ) increased following treatment application dates more so following the additions in 2001 when soil moisture and temperatures were high with infrequent periods of high rainfall (Figure 50 d).

Following stepwise multiple regression analyses (Minitab, USA) solar radiation, soil ammonium concentration, air temperature and rainfall were the significant factors controlling the variability of the nitrous oxide emissions. It is

interesting to note that soil moisture was rejected from the analyses; this could be due to the high spatial variability of soil moisture which was measured at the Sourhope field site as part of the Soil Biodiversity baseline data. Soil WFPS and soil nitrate concentration on the other hand were significant factors controlling the variability of nitric oxide flux following stepwise multiple regression analyses (Minitab, USA).



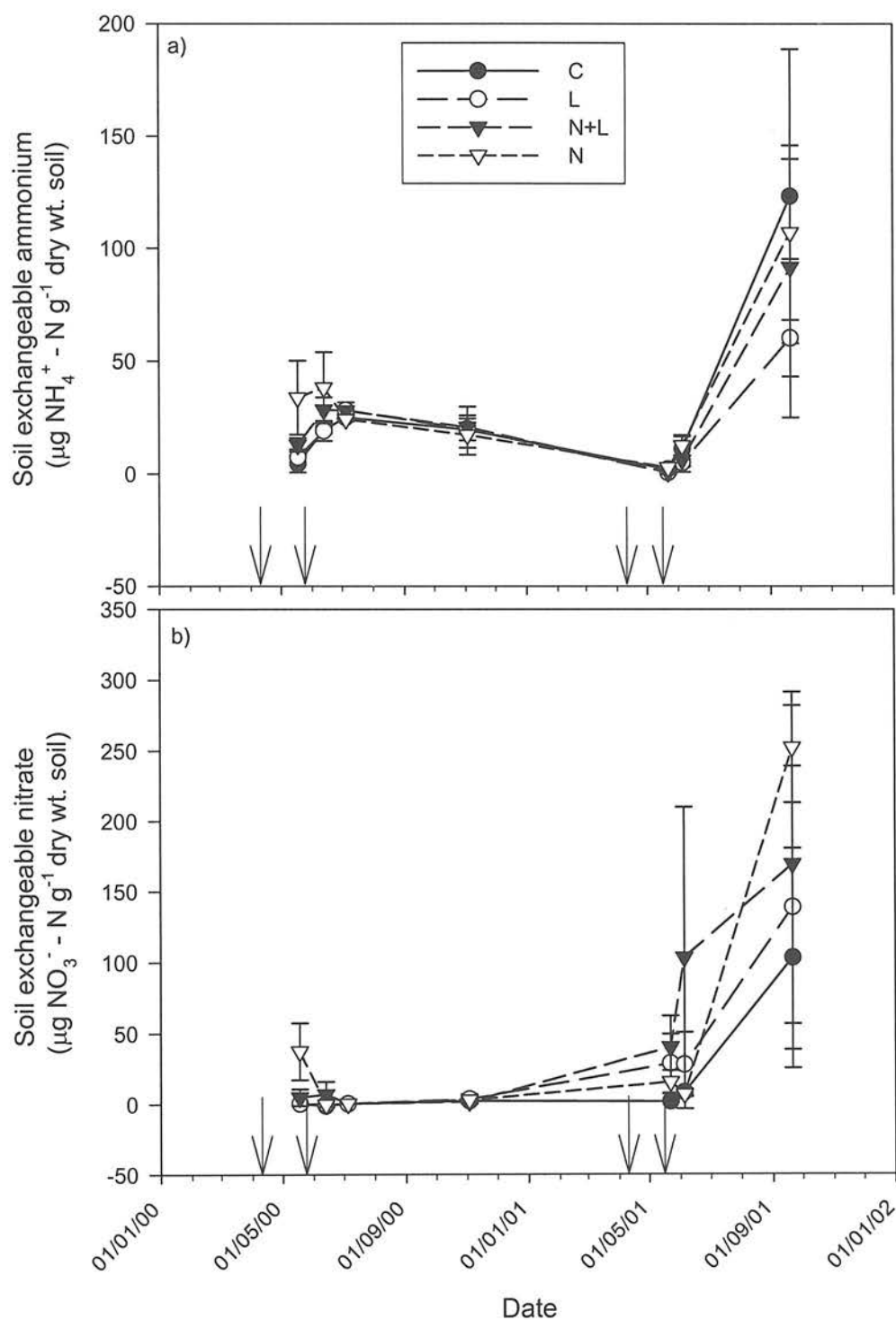


Figure 52. The effects of land management treatment applications in the field on soil exchangeable ammonium (a) and nitrate concentrations (b). Arrows indicate treatment application dates (C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$ , N+L =  $\text{NH}_4\text{NO}_3$  +  $\text{CaCO}_3$ )

The significant factors as determined by the stepwise multiple regressions for nitric and nitrous oxide were used in simple linear regression models to predict nitric and nitrous oxide emissions. The regression model for nitrous oxide is shown in Equation 16 and flux was calculated from total solar radiation ( $\text{MJoules m}^{-2}$ ), soil ammonium concentration ( $\mu\text{g NH}_4^+ - \text{N g}^{-1}$  dry wt. soil), air temperature ( $^{\circ}\text{C}$ ) and rainfall (mm). The regression model for nitric oxide is shown in Equation 17 and flux was calculated from soil nitrate concentration ( $\mu\text{g NO}_3^- - \text{N g}^{-1}$  dry wt. soil) and soil WFPS (%).

Equation 16. Stepwise linear regression equation for nitrous oxide emissions

$$\text{Flux } (\mu\text{g N}_2\text{O} - \text{N m}^{-2} \text{ h}^{-1}) = 0.318 + 0.0217 \times \text{solar radiation} + 0.00361 [\text{NH}_4^+] - 0.0618 \text{ air temp} - 0.0272 \text{ Rainfall}$$

Equation 17. Stepwise linear regression equation for nitric oxide emissions

$$\text{Flux } (\mu\text{g NO} - \text{N m}^{-2} \text{ h}^{-1}) = 0.509 + 0.0201 [\text{NO}_3^-] - 0.00549 \text{ WFPS}$$

The regression models were then used to predict nitric and nitrous oxide emissions from the field measurements using the predictor variables from Equations 16 and 17, the prediction field fluxes of nitric and nitrous oxide are shown in Figure 53 a and b respectively.

The predicted and actual emissions of both nitric and nitrous oxide were greater in the nitrogen and nitrogen + lime treatments, however when emissions of nitrous oxide were low in the field the regression model tended to overestimate the net flux by over 300%, with greater emissions from the control and lime treatments relative to the nitrogen and nitrogen + lime treatments. The regression model for

nitrous oxide emission was within  $\pm 60\%$  for the nitrogen + lime treatments when compared with the actual emissions. The correlation coefficient ( $r^2 = 0.417$ ,  $p < 0.001$ ) for the comparison between the measured and modeled fluxes of nitrous oxide was not very good but was in agreement with those from other studies (Clayton *et al.*, 1994), the correlation coefficient ( $r^2 = 0.473$ ,  $p < 0.05$ ) for the comparison between measured and predicted nitric oxide flux was also low.

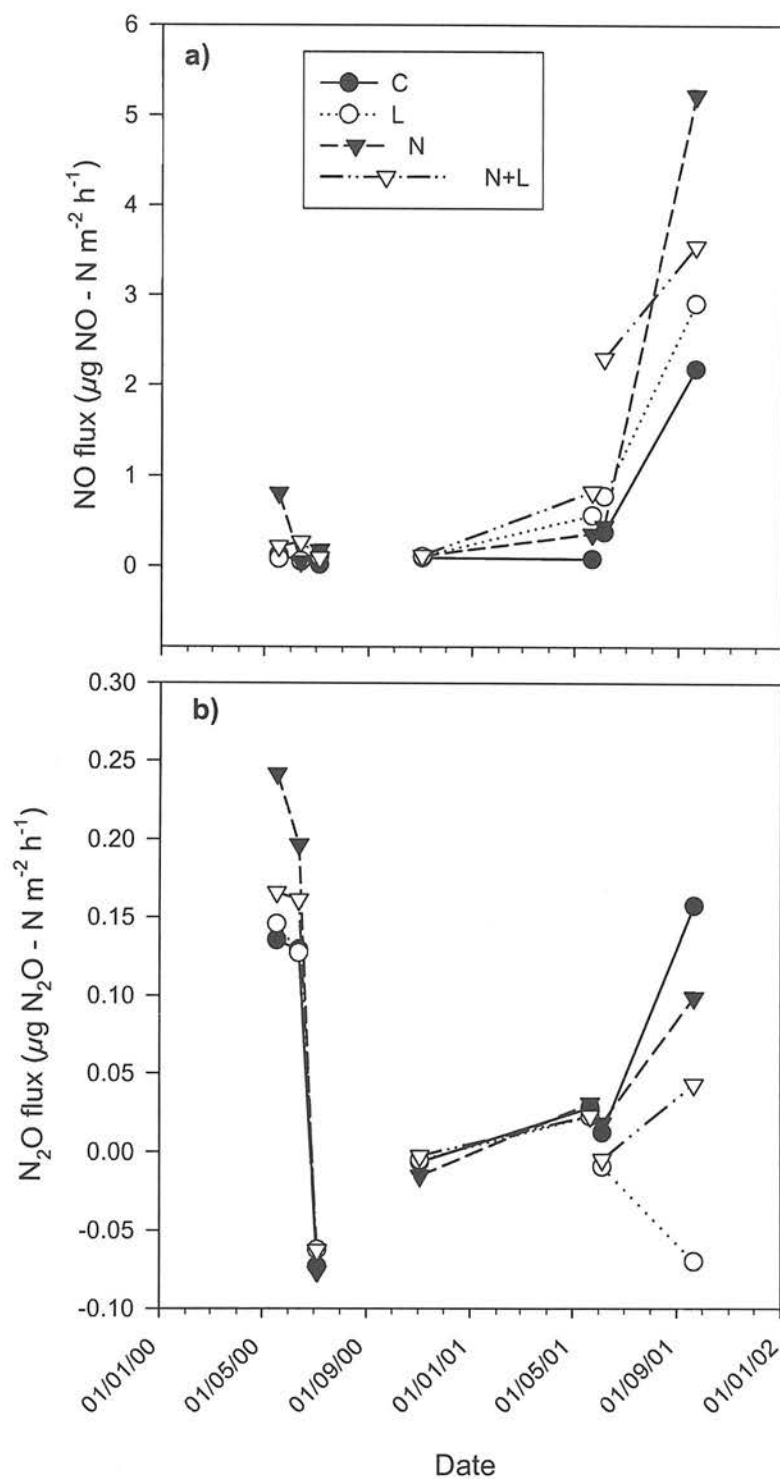


Figure 53. Regression model of nitric (a) and nitrous oxide (b) emission from an upland agricultural grassland soil following N addition indicated by the arrows (C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$  and N+L =  $\text{NH}_4\text{NO}_3 + \text{CaCO}_3$ )

### 7.3.4. Diurnal nitric and nitrous oxide flux

Diurnal measurements of nitric oxide were made during the 29<sup>th</sup> and 30<sup>th</sup> of June 2001 the results are shown in Figure 54 a and b for NO and NO<sub>2</sub> respectively. Samples were also taken for nitrous oxide, however due to equipment failure these data were lost.

Nitric oxide flux showed a clear diurnal pattern with very low fluxes during the night when air temperatures decreased rapidly from 20 to 12°C (Figure 54 c) and soil surface temperatures also decreased but only by a couple of degrees. The emission of nitric oxide increased to 7.9  $\mu\text{g NO} - \text{N m}^{-2} \text{h}^{-1}$  during the early morning (06:00). This increase in nitric oxide emission was only measured in the nitrogen treated plots, emission of nitric oxide from both the control and lime treated plots was approximately 0.2  $\mu\text{g NO} - \text{N m}^{-2} \text{h}^{-1}$  during the sampling period.

Changes in soil temperature were very small and only decreased by 1°C over the course of the sampling period. The rainfall over the two day sampling period was between 3 and 10mm on the 29<sup>th</sup> and 30<sup>th</sup> September respectively. The rainfall values were determined from the AWS at the field site and represent daily means.

There was a significant negative correlation between nitric oxide emission and soil temperature in the lime ( $r = -0.745$ ,  $p < 0.05$ ) and nitrogen ( $r = -0.784$ ,  $p < 0.05$ ) treatments only, there were no correlations between nitrogen dioxide and soil temperature for any of the treatments. Nitric oxide emission in the lime ( $r = -0.745$ ,  $p < 0.05$ ) and nitrogen ( $r = -0.784$ ,  $p < 0.05$ ) treatments also correlated with surface soil temperatures.

The flux of nitrogen dioxide was much more transient than that of nitric oxide and only increased at one measurement time in the early morning (06:00) on the 29<sup>th</sup> September. Again the flux only increased in the nitrogen and nitrogen plus lime treated plots.

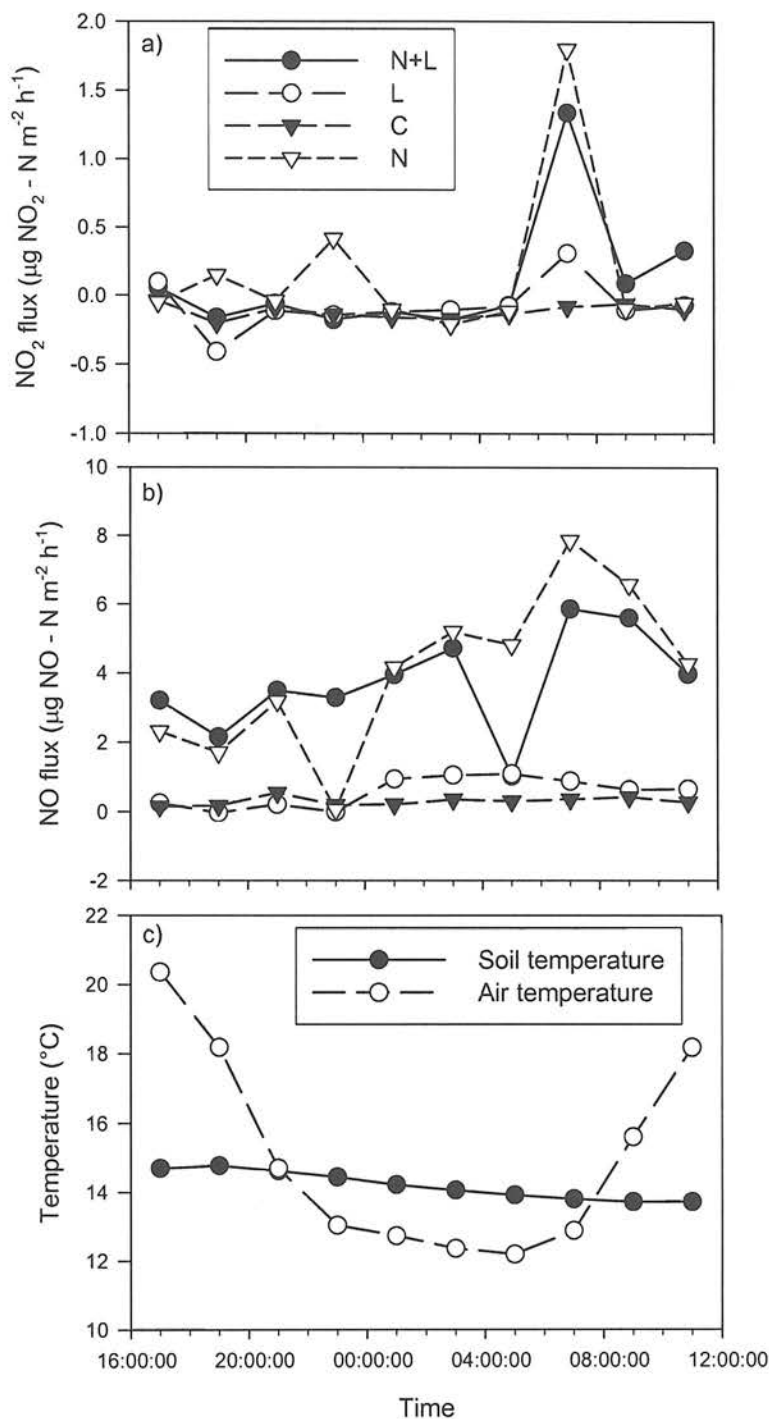


Figure 54. Diurnal field measurements of nitrogen di-oxide (a) nitric oxide (b) and soil and air temperature (c) over a 24 hour period from the 29th to the 30th September 2001 from treatment block 3 at Sourhope (C = control, L =  $\text{CaCO}_3$ , N =  $\text{NH}_4\text{NO}_3$ , N+L =  $\text{NH}_4\text{NO}_3 + \text{CaCO}_3$ ).



## 7.4. Discussion

Emissions of nitric and nitrous oxide increased following treatment application of nitrogen and nitrogen plus lime, nitric and nitrous oxide emissions were largest in the nitrogen plus lime treatment.

The largest emission of nitrous oxide was measured in the 2 weeks post fertiliser application during the 2000 measurement period, other studies in agricultural soils have also noted short lived increases in nitrous oxide emissions post fertiliser addition (Granli and Bøckman, 1994, Müller *et al.*, 2004), the flux of nitrous oxide is however comparatively low for a fertilised grassland soil (Skiba, 1996). Clayton *et al.*, (1994) observed nitrous oxide maxima within 5 days of fertiliser application in a fertilised grassland soil, which declined to 1/6 of their maxima within 3 weeks of N applications. The increased emissions in that study post N application was in part due to heavy rainfall just prior to additions (Clayton *et al.*, 1994).

Following stepwise multiple linear regression (Equation 16), rainfall was shown to significantly affect the emission of nitrous oxide. Clayton *et al.* (1994) found that soil nitrate and not ammonium, which was a more significant factor than nitrate in this study, was an important soil variable influencing nitrous oxide emissions along with rainfall and air temperature.

The regression model from this study and that of Clayton *et al.* (1994) predicted the emission of nitrous oxide well at when net emissions were high, with similar correlation coefficients, whereas the regression model of Venterea *et al.* (2000) tended to underestimate fluxes under low flux conditions and overestimate fluxes under high flux conditions. The emissions of nitrous oxide in the control and lime treatments were grossly overestimated in this study (difference between predicted and actual measured was >200% of the actual measured emission) under high flux conditions of high rainfall.

The importance of ammonium as determined by the stepwise linear regression in this study may indicate a different pathway for the formation of nitrous oxide compared with the study of Clayton *et al.* (1994), Wrage *et al.* (2001) have

identified nitrifier denitrification as another source of nitrous oxide in soils, in which autotrophic ammonium oxidisers are able to oxidise ammonium to nitrite followed by reduction of nitrite via nitric and nitrous oxide to  $N_2$ .

The conditions under which nitrifier denitrification occurs are, low oxygen concentrations coupled to low organic carbon content in soils and low pH (Wrage *et al.*, 2004). Given the low soil pH and high soil moisture contents with a significant correlation between nitrous oxide and soil ammonium concentrations, nitrifier denitrification may be another possible mechanism for the observed emissions of nitrous oxide under nitrogen and nitrogen plus lime treatments in these soils.

Nitrate was found to be a significant predictor for nitric oxide emissions along with soil WFPS following stepwise linear regression (Equation 17). Nitric oxide is an intermediate in the denitrification pathway, the emission of which is also strongly influenced by gas phase diffusivity which is related to soil moisture content. Under high soil moisture contents the diffusion of nitric oxide is limited and therefore the chances of further reduction to nitrous oxide and  $N_2$  by denitrifiers is increased. Nitric oxide is also produced as a by-product during nitrification of ammonium to nitrite under aerobic conditions.

However, since nitric oxide emissions were greatest when soil moisture contents were lowest, nitrification rather than denitrification is likely to be the source in this case, there was also a significant positive correlation between nitric oxide emission and soil nitrate ( $r^2 = 0.398$ ,  $p < 0.05$ ) but not soil ammonium concentrations.

Using  $^{15}N$  kinetic isotope methods (KIM), Russow and Neue (2000) quantified the emission of both nitric and nitrous oxide via nitrification and denitrification from a fine-silty loess-black earth soil, they noted that emission of nitric oxide via denitrification did not occur in that soil type despite anaerobic conditions and was predominantly due to nitrification of ammonium to nitrite.

The correlation between nitric oxide emissions and soil nitrate may be a reflection of ammonium oxidation via nitrification to nitrate, and would be in agreement with the model proposed by Firestone and Davidson (1989) with increased nitric oxide emissions at increasing soil nitrate reflecting an increase in

ammonium oxidation and nitrate accumulation. The accumulation of nitrate following ammonium fertiliser application has been shown by Abbasi and Adams (2000) in a grassland soil at low soil moisture contents (63%), in this study nitrate accumulation occurred at soil WFPS between 60 and 70% WFPS agreeing with the findings of Abbasi and Adams (2000).

The control and lime treatments were either a net sink or small source of nitrous oxide over the sampling period from 2000 to 2001. This in comparison with nitrogen and nitrogen plus lime treatments, in which nitrous oxide emissions were as high as  $0.4 \mu\text{g N}_2\text{O} - \text{N m}^{-2} \text{ h}^{-1}$  could have significant implications for mitigation strategies in upland agricultural grasslands.

Regression modeling of nitrous oxide emissions however, predicted greater emissions from both the control and lime treatments indicating that factors other than those measured and modeled could be affecting emissions, for example soil pH which has been shown to influence emissions of both nitric and nitrous oxide (Šimek and Cooper, 2002). Lime additions have also been found to affect soil structure of organic layers resulting in increased leaching of soil DOC (Broken and Brumme, 1997; Andersson and Nilsson, 2001), which could increase denitrification and potential nitrous oxide respiration. The mechanisms for nitrous oxide uptake may include respiration by heterotrophic bacteria including denitrifiers. The production of nitrous oxide at deep soil layers is often consumed by denitrifiers resulting in low net emissions despite high production rates (Müller *et al.*, 2004), therefore net uptake of nitrous oxide could reflect sub optimal conditions for denitrification since nitrous oxide reductase is not as susceptible to oxygen as the other denitrification enzymes.

The diurnal field flux measurements of nitric oxide emissions were greatest after sunrise. Nitric oxide is produced predominantly via nitrification under aerobic soil conditions where  $\text{NO}:\text{N}_2\text{O}$  ratio's are generally greater than unity (Lipschultz *et al.*, 1981). The increased nitric oxide emissions in the nitrogen and lime treatments were negatively correlated with both soil surface and air temperature, this is in contrast to the findings of Skiba *et al.*, (1997) who reviewed several studies of nitric oxide emissions and found that nitric oxide was positively correlated with soil

temperature and soil nitrate, however these studies did not measure diurnal emissions.

From the regression equation for nitric oxide emissions soil temperature was not found to be an important factor controlling emissions, which could be explained by the significant small temporal effects of temperature on nitric oxide in the diurnal field measurements. In another study of diurnal nitric oxide emissions Skiba *et al.* (1992) also found significant negative correlations between soil air and surface temperatures in an agricultural sandy loam soil with greatest emissions during the early morning.

This diurnal pattern of nitric oxide in which emissions are greatest in the early morning at sunrise, may be due to rapid surface drying effects of increased air temperature on the top couple of cm of soil, Skiba *et al.* (1992) attributed the soil surface as the source of nitric oxide production during similar diurnal field measurements.

The effects of treatment on the diurnal measurements were consistent with those made throughout 2000 and 2001 field campaigns, with largest emissions from the nitrogen and nitrogen + lime treatments. All field measurements of nitric oxide were made at or around mid day which corresponds to the average emission during the diurnal field measurements.

An increase in the NO<sub>2</sub> emission was measured at 06:00 which corresponded to maximum emissions of nitric oxide, this may be due to nitric oxide reactions with ozone producing NO<sub>2</sub>.

The land management strategies used in this upland grassland soil resulted in increased emissions of both nitric and nitrous oxide following addition of inorganic N alone and in combination with lime. The timing of fertiliser application was critical in determining the species of N lost as gas, this corresponded to the rainfall and WFPS being significant factors for the emissions of nitric and nitrous oxide respectively. Nitrous oxide emissions only significantly increased following periods of rainfall after fertilizer addition, whereas nitric oxide emissions were greatest following periods of low rainfall.

This upland soil is a potential sink for nitrous oxide under non managed regime or with lime additions only; this has significant implications for future mitigation and farming practices in upland soils and potentially the as yet unknown sink size for nitrous oxide in soils.

## Chapter Eight. General Discussion

### 8.1. Main findings

- The emission of nitric oxide is significantly affected by soil moisture, with increased NO:N<sub>2</sub>O at low soil WFPS, although overall NO:N<sub>2</sub>O ratio's were much greater than those reported previously.
- The conceptual model of Davidson for gaseous N emissions and nitric and nitrous oxide flux was only able to explain the nitric oxide emissions in these soils with the nitrous oxide emissions increasing only at 80% WFPS. The model of Davidson predicts increased nitrous oxide at 50 to 60% WFPS followed by N<sub>2</sub> production at soil WFPS above 70%. The emissions of nitric oxide in this study were significantly negatively correlated with soil moisture whereas nitrous oxide emissions only increased under saturated soil moisture conditions. In these soils N loss via NO and nitrification could potentially be more significant than N<sub>2</sub>O loss via anaerobic microbial processes such as denitrification. This study provides one of the few experimental data sets to test the model proposed by Davidson (1991).
- The <sup>15</sup>N pool dilution measurements of gross nitrification correlated with increased nitric and nitrous oxide emissions. These data agree with the hole in the pipe model of Firestone and Davidson (1989) in which increased process rates of N cycling resulted in increased N loss as nitric and nitrous oxide.
- Effects of soil temperature, indicated optima for nitric and nitrous oxide emissions at different temperatures at low and high soil WFPS, this was one of the few studies looking at the direct effects of soil temperature on emissions of nitric and nitrous oxide in upland agricultural grassland soils.
- Following incubation experiments in controlled soil microcosms with land management treatments, addition of lime to this acidic upland soil resulted in changes in the eubacterial microbial populations and subsequent increased nitric and nitrous oxide emissions. This putative link between a microbial



function and community structure indicates that microbial diversity may be important for ecosystem stability.

- In general the number of DGGE bands extracted from soil incubation experiments either increased or did not change relative to the control soil for all three functional groups measured as a result of the different treatments. The *Nitrobacter* species DGGE profiles were strongly influence by lime, whereas AOB populations did not change in terms of DGGE banding patterns with treatment.
- The variability in the gross nitrification rates measured in chapters 5 and 6 indicate that the pool dilution methods used in this study could be improved, particularly with the application of labelled nitrogen in intact previously re-packed soil cores.
- Field fluxes of both nitric and nitrous oxide were low, except immediately after fertiliser and lime additions with increased nitric oxide flux under dry soil conditions and nitrous oxide following rain events in agreement with other studies.
- There is potential for net uptake of nitrous oxide in un-improved upland soils which have either had no previous history of land management or that have received only lime. This is important for the mitigation of gaseous N losses in upland agricultural systems, particularly so given that nitrous oxide is a greenhouse gas.
- The environmental terms important for nitric and nitrous oxide emissions in the DNDC model and those identified from the linear regression modelling in this study are similar in their most basic form; however, actual and predicted emissions in the control and lime treatments for nitrous oxide using the regression equation 16 in this study resulted in a net emission as apposed to net uptake of measured nitrous oxide. This could have significant implications for IPCC emission scenarios, and could help to explain some of the source sink imbalance for nitrous oxide in soils.

## 8.2. Effects of environmental conditions on nitric and nitrous oxide emissions

The results presented in this thesis highlight the importance of environmental factors that control emissions of nitric and nitrous oxide. The effects of soil moisture and temperature on nitric and nitrous oxide production shown in soil microcosms were also found to be important factors affecting emissions of nitric and nitrous oxide in the field flux studies.

These data fit the model proposed by Davidson (1991) for nitric oxide emissions, with a linear decrease in emission with increasing soil moisture content. Other studies have shown that the NO:N<sub>2</sub>O ratio decreases with increasing soil WFPS (Smith *et al.*, 2003). The effects of soil WFPS on the NO:N<sub>2</sub>O ratio are presented in Figure 55, the log<sub>10</sub> of the NO:N<sub>2</sub>O ratio's in these data are much greater than those reported by Smith *et al.* (2003) ranging from 4 to 2 at 30 and 80% WFPS respectively compared with 1 and -2 at 30 and 80% WFPS respectively (Smith *et al.*, 2003). The contribution of nitrification to nitrous oxide emissions has already been shown to be in excess of 60% of the total emissions following analyses of the <sup>15</sup>N enrichment of nitrous oxide following pool dilution, therefore the increased emissions at high soil WFPS are probably due to denitrification. The nitrous oxide emissions in the Sourhope treatments were only significant above 80% WFPS in both lab and field studies in the nitrogen and nitrogen plus lime treatments. The effects of soil moisture on the flux of both gases are probably mediated via effects of gas diffusion, substrate availability and the microbial processes themselves. Also in the soil inhibition experiments at 60% WFPS nitrification accounted for more than 99% of the nitrous oxide emissions in all treatments.

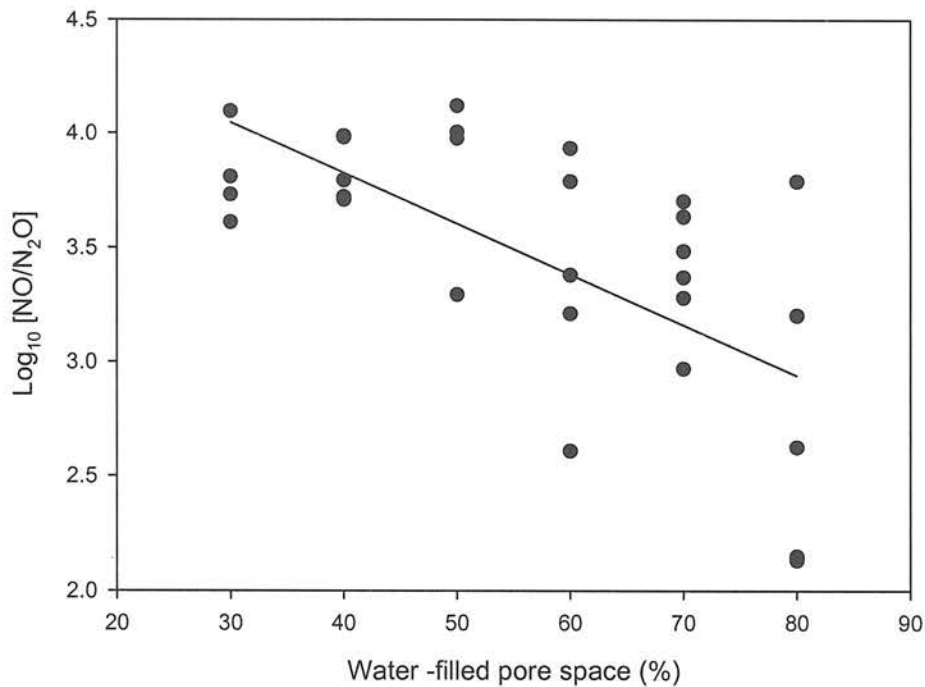


Figure 55. Relationship between soil WFPS and the ratio of NO emissions to N<sub>2</sub>O emissions for fluxes measured in chapter 4. The solid line is the regression ( $R^2 = 40.9\%$ ,  $P < 0.0001$ ).

The enzymes in the denitrification pathway especially the nitrate reductase are sensitive to oxygen tension with different NIR enzymes expressed under different oxygen concentrations (Zumft, 1997; Richardson and Watmough, 1999), this would result in different metabolic rates for nitric and nitrous oxide production with the different enzymes due to different  $K_m$  values for nitrate reduction, ranging from 0.3 to 3.8 mM (Zumft, 1997), the high sensitivity of denitrification enzymes may also explain the small contribution to nitrous oxide emissions below 80% WFPS in the controlled soil moisture experiments.

The autotrophic nitrifiers are inhibited by low oxygen concentrations with reduced nitrification rates; this combined with higher  $K_s$  values for oxygen when compared with heterotrophs (Prosser, 1989). Soils in which heterotrophic

nitrification is already dominant due to low soil pH and high C:N ratios (Hagopian and Riley, 1998) (as is the case in the Sourhope treatments not treated with lime) would mean that autotrophic nitrifiers would be out competed by heterotrophs for inorganic N and oxygen at increasing soil moisture contents if organic carbon was readily available.

Under dry field conditions nitric oxide fluxes could be a significant loss of nitrogen following fertiliser application in this soil, previous studies have related this to rates of nitrification (Skiba *et al.*, 1992).

Nitric oxide emissions were always greater than nitrous oxide emissions even under favourable conditions for nitrous oxide production. The emission of both nitric and nitrous oxide in these data would indicate that nitrification and denitrification under aerobic and anaerobic conditions respectively are responsible for these fluxes, although G  dde and Conrad (1998) noted that with increasing soil moisture content the % contribution of denitrification to NO flux did not increase, and that NO flux even at high soil moisture contents in agricultural soils with similar pH ( $\approx 5$ ) and C:N ratios (13) as those presented here were mainly due to nitrification.

These data, where nitrate reduction at low soil moisture content was high and fluxes of nitrous oxide low in the acidic soils could indicate that denitrifier nitrification may be another possible source of these gases (Ruscow *et al.*, 2000).

The flux of nitric and nitrous oxide decreased following initial N additions at all soil moisture contents, this was coupled to a decrease in the soil ammonium pool with time at 30% WFPS and an increase in the soil nitrate at 80% WFPS; this would indicate that nitrification is the dominant process at low soil moisture contents where inorganic ammonium is reduced to nitrate with nitric oxide produced as a by-product of ammonium oxidation to nitrite (Ruscow *et al.*, 2000); and that denitrification (predominantly an anaerobic process) is a dominant source of nitrous oxide at high soil moisture contents.

One of the IPCC strategies for mitigating nitrous oxide emissions from agricultural land includes the timing of fertiliser application to coincide with periods of low rainfall (IPCC, 2001). This is based on the DNDC model of Li (2000) in

which modelled nitric and nitrous oxide emissions via nitrification take into account soil ammonium concentrations, pH, nitrifier biomass, and temperature. The emissions of nitric and nitrous oxide via denitrification take into account soil porosity, moisture, temperature and clay content as well as soil nitrate concentration.

Given that the DNDC model is in fact much more complex than the simple terms used to quantify emissions of nitric and nitrous oxide, the terms from the stepwise multiple regressions in this study do correlate with some of the terms in the DNDC model. These data can be used for further validation for the DNDC model (Li, 2000) to help predict nitric and nitrous oxide emissions from soils as a result of changing soil moisture to improve global estimates for their emissions. Groffman and Tiedje (1991) found significant correlations between soil air filled pore space and denitrification rates which were opposite to and complimentary to the significant correlations between nitric oxide flux and soil WFPS presented in this thesis.

The effects of soil temperature on the microbial production of nitric and nitrous oxide were different at two different soil moisture contents. Results from these data indicated temperature optima for nitric oxide production at low soil WFPS (40%), predominantly produced via nitrification, was 5 - 15°C. The mean yearly average soil temperature in the field is 10°C with a soil WFPS of 40-50%, it is therefore not surprising that under low soil WFPS the production of nitric oxide occurs at a maximum soil temperature similar to that of the field site and that the apparent  $Q_{10}$  values for the emission of nitric oxide were also greatest over this temperature range at 40% WFPS.

This optimum would suggest some predominant adaptation to low soil moisture contents, adaptation by microbial populations to the environmental conditions in which they are found has been well documented (Atlas and Bartha, 1998); and forms the basis for natural selection and species diversity, which applicable to all species on this planet (Darwin, 1859)

The maximum nitric oxide flux at 80% WFPS was 25°C. This was in contrast to that at 40% WFPS, the two different maxima for nitric oxide flux may represent maxima for nitrifier and denitrifier production or simply effects of drying and decreasing soil moisture content at higher temperatures. Despite increasing nitric

oxide emissions with increasing temperature at 80% WFPS the apparent  $Q_{10}$  values were not significantly different. Increasing nitric oxide flux with temperature at 80% WFPS could therefore be the result of drying effects due to higher soil temperatures,

As the soils dry out the top centimeter would provide optimal conditions for nitrification with low soil moisture content and high soil ammonium concentrations as a result of initial N additions, data presented here have already shown maximum nitric oxide emissions at 30 to 40% WFPS.

The effects of soil temperature on the nitrous oxide flux were not as clear as those on nitric oxide flux. However it appeared that highest fluxes were at 15°C at low soil WFPS corresponding to the maximum nitric oxide flux. The apparent  $Q_{10}$  values were also greatest at the lower soil temperature ranges for nitrous oxide emissions, between 1 to 10 ( $Q_{10}$  2.43) and 5 to 15°C ( $Q_{10}$  2.16). These data are similar to those for other grassland soils in which nitrous oxide emissions were greatest at 18°C following fertiliser and water addition to soil cores (Dobbie and Smith, 2001) with  $Q_{10}$  values of 3.7 and 2.3 over 5 – 12 and 12 – 18°C temperature ranges.

The effects of soil moisture on gas transport have been reported previously (Davidson and Schimel, 1995) and in an open system it is likely that higher temperatures did result in “artificially” enhanced emissions of nitrous oxide due to increased diffusion out of the soil rather than true temperature effects on microbial processes. Optimum temperatures for nitric and nitrous oxide indicated different maxima at low and high soil WFPS, this may be complicated by the effects of drying but could also indicate two different processes were responsible for emissions at 40 and 80% WFPS.

#### **8.4. Effects of nitrogen and lime on autotrophic and heterotrophic nitrification**

These data show that land management treatments used in this study have an affect on the production of nitric and nitrous oxide which may be mediated via changes in soil chemistry as measured by soil pH. Increasing the pH had little effect on the gross nitrification rates but did result in decreased nitrous oxide emissions.



The use of specific inhibitors to distinguish between heterotrophic and autotrophic nitrification as sources of nitric and nitrous oxide did not clearly indicate dominance of either process. However it does highlight the difficulties in using specific inhibitors that are not applied as a gas such as acetylene, addition of an even spray of inhibitor at the microbial scale to soil microcosms is probably impossible to achieve and large variation between treatment replicates here would confirm that.

The use of acetylene at 0.01% v/v to inhibit the AMO enzyme of autotrophic nitrification would be better than application of DCD as a spray at low soil moisture contents (Inubushi *et al.*, 1996), however the efficiency of inhibition by acetylene at increasing soil moisture contents would have to be tested for this soils type.

The field site has a low pH (4.54-4.81) this has been shown to favour heterotrophic nitrifiers more than autotrophic (Hagopian and Riley, 1998). Following the addition of lime and nitrogen to control plots in the field since 1998 the soil pH was measured in soil sampled from the different treatments prior to these experiments. The four Sourhope soil treatments of control, lime, nitrogen and nitrogen plus lime had a soil pH of 4.76, 5.6, 5.16 and 5.24 respectively; long term fertilisation with ammonium based fertilisers and lime has already been shown to result in decreased and increased soil pH respectively (Parkin *et al.*, 1985).

There were no significant differences in the gross nitrification rates between the uninhibited treatments relative to the control treatments therefore increasing the pH had little effect on the gross nitrification rates but did result in decreased nitrous oxide emissions.

When the effects of inhibitor on the Sourhope soils were compared with those of the Beachgrove soil it was clear that nitrification rates were very low in this upland agricultural grassland even in the nitrogen amended Sourhope treatments.

Following the use of the inhibitor DCD and  $^{15}\text{N}$  pool dilution techniques, gross nitrification rates increased in all soil treatments at all soil pH's with the exception of the nitrogen treatments; this indicates that heterotrophic nitrification rates are greater than autotrophic nitrification rates in these soils but are out competed by autotrophs for ammonium in uninhibited soils. In pure cultures of

*Nitrosomonas europaea* and *Alcaligene faecalis* an autotrophic and heterotrophic nitrifier respectively, Anderson *et al.* (1993) measured nitric and nitrous oxide emissions, with 10 fold more nitrous oxide produced per cell by *A. faecalis* than *N. europaea* indicating greater rates of nitrification in heterotrophic bacteria which are comparable to the measured increases in the emission of nitrous oxide with DCD inhibition.

With no effect of nitrogen addition on the gross nitrification rates between DCD and non DCD inhibited soils would indicate that either autotrophs are not nitrifying in these soils or that DCD had no effect in this treatment. With increased emissions of nitrous oxide in the nitrogen and control treatments compared to lime and nitrogen plus lime treatments possibly heterotrophic nitrification can result in higher emissions than autotrophic. The inhibition of either autotrophic or heterotrophic nitrification using DCD or cyclohexamide respectively, results in reduced competition for ammonium. The autotrophic nitrifiers have been shown to have lower  $K_m$  values for ammonium than heterotrophic nitrifiers (Stark and Firestone, 1996) (k and r strategists respectively), these data show that in the treatments without DCD gross nitrification rates were lower and autotrophic nitrifiers more active, conversely in DCD amended treatments where autotrophs were inhibited, and therefore heterotrophs resulted in increased gross nitrification rates.

### 8.5. Soil long term incubation and microbial diversity and function

Following long term incubation of soil microcosms with the different Sourhope land management regimes, emissions of nitric and nitrous oxide increased in the nitrogen and nitrogen + lime treatments, which was also measured in the field.

The Sourhope treatment additions did result in changes in function and also subsequent changes in microbial community structure and was emphasised in the nitrite and lime treatments with changes in heterotrophic eubacterial DGGE banding patterns. The treatment effects on the changes in the specific microbial functional groups as determined by the DGGE banding patterns also showed that lime, alone or in combination with nitrogen or nitrogen plus acetate increased the number of

eubacterial microbial species relative to non amended control treatments. The changes in eubacterial DGGE banding profiles in the lime treatments may be associated with changes in function; where the nitric to nitrous oxide ratio was greater in the lime treatments than non limed treatments (Figure 46 a) which was also associated with increased gross nitrification rates (Šimek and Cooper, 2002).

Liming has been used extensively in forest soils to minimise the effects of acid rain deposition on parts of Sweden and so would raise the soil pH, it is also a commonly farming practice within the UK. Soil pH has previously been shown to significantly affect distribution patterns of autotrophic nitrifier strains isolated from agricultural soils (Koops and Pommerening-Roser, 2001). A review of nitrification in acid soils by De Boer and Kowalchuk (2001) indicated that autotrophic rather than heterotrophic nitrification was the dominant process in terms of nitrogen cycling in most acid soils, although the contribution by heterotrophic nitrification is difficult to assess due to their functional diversity for example the nitrifying denitrifiers (Robertson and Kuenen, 1991).

From these data land management with addition of lime could increase the autotrophic nitrification rates at Sourhope. This has implications for ecosystem resilience and gaseous nitrogen losses; autotrophs tend to grow much more slowly (Prosser, 1989) and require inorganic nitrogen either from mineralisation or N input for energy. Autotrophs are also less able to compete for N than heterotrophic nitrifiers or plants.

The addition of acetate as a simple organic carbon source enhanced nitrous oxide production and nitric oxide flux at low N application rates, but not at the 21 week application rate. Soils amended with glucose have been shown to exhibit increased rates of denitrification; for example Lind and Eiland (1989) detected significant increases in nitrous oxide production in sandy-clayey soil amended with organic. The interactions between plant species diversity and below ground microbial processes may also play an important role in driving below ground function in upland grassland soils (Grayston *et al.*, 2004), Dawson *et al.* (2004) quantified root turnover rates and carbon and nitrogen input to soil at the Sourhope Soil Biodiversity

field site, in the nitrogen plus lime amended plots they noted increased N and C input to the soil rhizosphere relative to control plots which corresponded to increased emission of nitric and nitrous oxide in this study.

The use of stable isotope ratios for discriminating between sources of nitrous oxide has been studied extensively at natural abundance levels (Bergsma *et al.*, 2001; Stevens *et al.*, 1998; Stevens and Laughlin, 1998; Bergsma *et al.*, 1999). The application of these methods at enriched levels here was used to partition nitrous oxide production between nitrification and denitrification. The nitrogen, nitrogen plus acetate, nitrogen plus lime and nitrite treatments had low atom%  $^{15}\text{N}$   $\text{N}_2\text{O}$  similar to that of the ammonium atom %  $^{15}\text{N}$ , following further analyses of the  $^{15}\text{N}_2\text{O}$  data the proportion of nitrous oxide produced via nitrification and denitrification was quantified, although there were no significant differences between treatments, nitrification as a source of nitrous oxide was greater in lime and nitrogen amended soil microcosms.

## 8.6. Effects of land management on the emissions of nitric and nitrous oxide in the field

The fluxes of nitric and nitrous oxide both increased following treatment application in the nitrogen and nitrogen plus lime plots, nitric and nitrous oxide fluxes were greatest in the nitrogen and lime treatments. The effects of lime also resulted in increased nitric and nitrous oxide production in both long term enrichment and inhibition studies. With inhibition of autotrophic nitrification greater in lime amended soils than in unlimed soils it would indicate that liming results in increased autotrophic nitrification and also increased N losses relative to those via predominantly heterotrophic nitrification in nitrogen treated plots.

The effects of soil moisture determined the species of N gas evolved following treatment applications; under high soil moisture contents and therefore more anaerobic conditions very low nitric oxide fluxes and high nitrous oxide fluxes were measured in the nitrogen and nitrogen plus lime treatments. These findings were similar to those made in controlled soil microcosm experiments where nitric

oxide flux was greatest below 40% WFPS and nitrous oxide only increasing above 80% WFPS with ratios of NO:N<sub>2</sub>O negatively correlated with increasing soil WFPS.

The WFPS model from Davidson (1991) shown above could explain the relatively high fluxes of nitrous oxide at high soil moisture contents coupled to low nitric oxide fluxes as being produced via denitrification. The flux of nitrous oxide here is comparatively low for a fertilised grassland soil (Skiba, 1996), which may be due to nitrous oxide reduction to N<sub>2</sub> via denitrification at high soil WFPS (Zumft, 1997), although no <sup>15</sup>N<sub>2</sub> was detected following the <sup>15</sup>N pool dilution incubation experiments in soil microcosm experiments, the soil WFPS was never greater than those measured in the field however and therefore complete denitrification to N<sub>2</sub> was unlikely.

Following stepwise multiple regression analyses of the field flux measurements for both nitric and nitrous oxide, rainfall soil ammonium concentration and air temperature were significant factors affecting the emission of nitrous oxide whereas soil WFPS and soil nitrate concentrations significantly affected emissions of nitric oxide. The effects of soil moisture content expressed as WFPS on the emissions of nitric oxide in the field were significantly correlated with each other in the soil moisture microcosm experiments, soil WFPS is also used in many of models used to predict N cycling processes and gaseous emissions, the DNDC model being one of them (Li, 2000).

The model of Davidson is purely conceptual although based on experimental observations, it is clear emissions of nitric and nitrous oxide will be difficult to predict for all soil types and habitats using just one model. The results from this study provide yet more evidence that important variables controlling emissions of nitric and nitrous oxide are moisture either as WFPS or rainfall, temperature and soil ammonium and nitrate concentrations, however it does highlight the importance of measuring these effects experimentally so that the conceptual models of Davidson (1991) and Firestone and Davidson (1989) can be optimized and tested to help predict future emissions estimates more accurately using the more complex DNDC model (Li, 2000).

In acidic soils nitric and nitrous oxide emissions via nitrification and denitrification may not be clear cut and other novel processes such as nitrifier denitrification (Wrage *et al.* 2001) and anaerobic ammonium oxidation (Jetten *et al.*, 1997) also need to be quantified and included in future models.

This study enhanced our understanding of the source/sink strength of nitric and nitrous oxide emissions in upland grassland soils and the potential net emissions from those soils following fertilizer additions. Current sink sizes for nitrous oxide in soils are unknown (Mosier *et al.*, 1998), since the IPCC estimates only take into account anthropogenic emissions of nitrous oxide (i.e. N application) the potential for soils to act as a net sink either has not been extensively studied or deemed to be insignificant. The net uptake of nitrous oxide over a 2 year period in the control and lime treatments in this upland grassland is a significant finding and should help to improve IPCC estimates of soil sinks for nitrous oxide and drive policy for management practices in upland grasslands.



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## Appendix 1

The influence of land-use management practices on species and functional biodiversity of nitrite oxidising bacteria and nitrification and denitrification processes.

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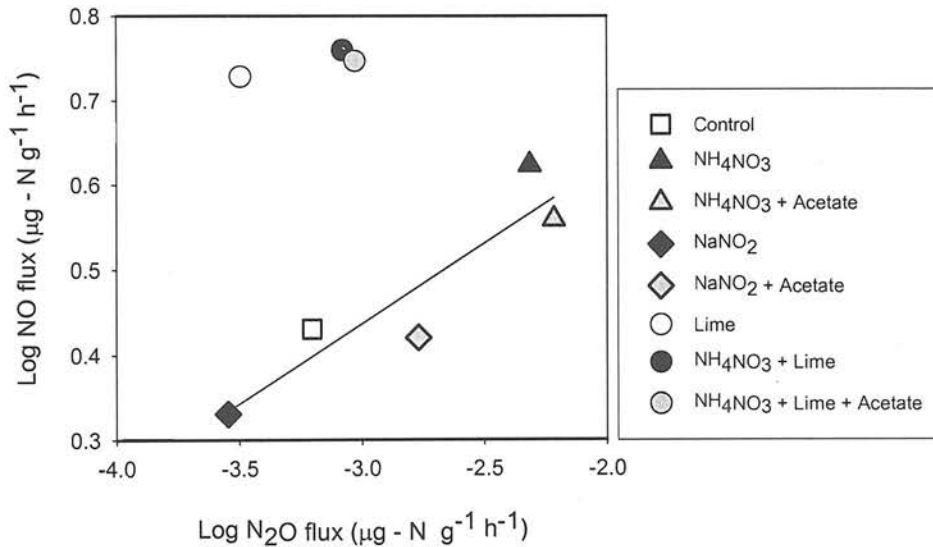
Davies, C.A., Speksnijder, A.G.C.L., Smith Z, Brown, H.L., Skiba, U.M., Embley, T.M., Rees, R.M., McCaig, A.E. & Prosser, J.I.

Efficient functioning of soil ecosystems relies on the biogeochemical cycling of nitrogen. The major inorganic pools of nitrogen, ammonia and nitrate, are determined by two key processes within the soil nitrogen cycle: nitrification, the sequential oxidation of ammonia to nitrite and nitrate, and denitrification, the reduction of nitrate to gaseous products. These processes control nitrogen supply to plants, losses of gaseous nitrogen, leaching of nitrate and generation of greenhouse gases. This project focuses on the organisms responsible for the second stage of nitrification, the nitrite oxidising bacteria. These organisms convert nitrite to nitrate under aerobic conditions, but under anaerobic conditions can reverse the process, reducing nitrate. Little is known of their diversity and the aim of the project was to use a combination of molecular techniques for analysis of bacterial diversity and microcosm and field determination of nitrogen cycle process rates, to determine the influence of soil management processes on their community structure and on the rates of processes in which they were involved.

### Microcosm studies

Soil moisture strongly influenced emissions of NO and N<sub>2</sub>O. NO emissions peaked under the driest soil conditions, whereas N<sub>2</sub>O peaked in the wettest soils. Autotrophic nitrification inhibitors indicated that autotrophic nitrification contributed 12% of the NO flux, suggesting significant heterotrophic nitrification at Sourhope. Long-term enrichment of Sourhope soil showed that lime additions, combination with other treatments, increased the emission of NO relative to N<sub>2</sub>O (Fig. 1a) and also increased molecular diversity (Fig. 1b). The contribution of denitrification to the N<sub>2</sub>O flux was largest in the lime-treated soils; however complete denitrification to N<sub>2</sub> was not detected (<sup>15</sup>N studies). The distribution of <sup>15</sup>N in the N<sub>2</sub>O molecules suggested that more than one process was responsible for N<sub>2</sub>O production.

Figure 1a. The influence of long term enrichment of NH<sub>4</sub>NO<sub>3</sub>, lime, acetate and NaNO<sub>2</sub> on the emissions of NO and N<sub>2</sub>O. The regression line was fitted to the non-lime treated soils only ( $r^2 = 0.75$ ).





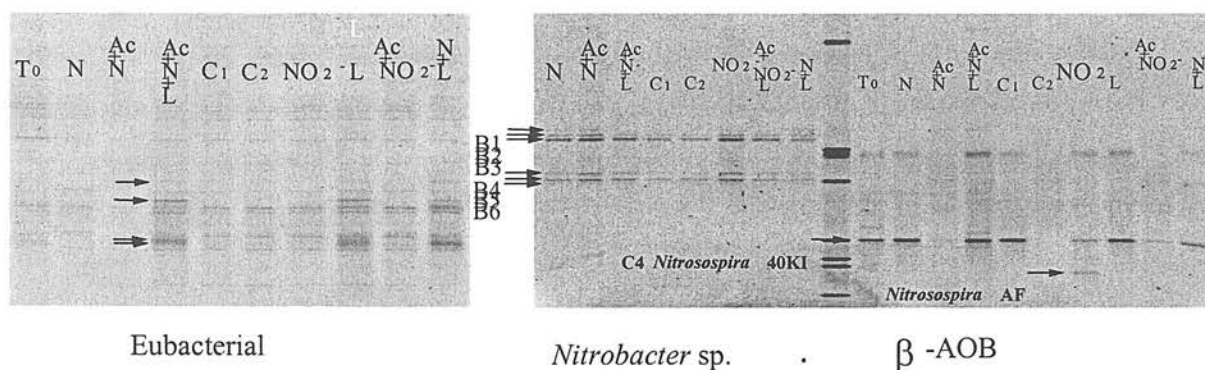


Fig. 1b. DGGE analysis of 16S rRNA genes amplified from extracted DNA using eubacterial, *Nitrobacter* and AOB primers. C – control, N – nitrogen, L – lime, Ac – acetate, T<sub>0</sub> – initial value.

Bacterial communities were characterised by analysis (denaturing gradient gel electrophoresis; DGGE) of 16S rRNA genes amplified from extracted nucleic acids using primers specific for eubacteria, ammonia and nitrite oxidisers. This indicated differences between eubacterial communities in microcosms treated with lime and with nitrogen and organic carbon. Nitrite oxidiser communities were dominated by *Nitrobacter*-like sequences and profiles derived from *Nitrobacter* primers were similar in different treatments, but with some evidence of increases in some sequence types following nitrogen amendment. Ammonia oxidisers were dominated by *Nitrosospira* 40KI-like sequences, with *Nitrosospira* species AF-sequences increasing following nitrite amendment. Analysis of 16S rRNA genes from enrichment cultures of nitrite oxidisers indicated similarities with those amplified directly from soil but none was identical to sequences from laboratory cultures, with the exception of *Nitrobacter* sp. ATCC25325.

## Field studies

Emissions of  $\text{N}_2\text{O}$  and  $\text{NO}$  and soil mineral N concentrations increased after N fertilisation with ammonium nitrate, with further increases in emissions in nitrogen and lime treated soils. Treatment effects were short lived and after 8 weeks gaseous loss of  $\text{NO}$  and  $\text{N}_2\text{O}$  was close to that of the control plots. Molecular analysis of ammonia oxidiser and *Nitrobacter* communities in field plots indicated a reduction in heterogeneity following soil amendment but little variation in *Nitrobacter* profiles between plots.

## **Appendix 2**

### **Sourhope Field Experiment**

#### **Report IV (1999 – 2002)**

**Written by Graham Burt-Smith**

## SUMMARY

It is now four years since the establishment of the NERC Soil Biodiversity Thematic Programme, centred upon the intensive study of a large field experiment located at the Macaulay Land Use Research Institute's farm at Sourhope in the Scottish Borders. During this time, the site has been monitored to assess changes in aboveground biomass production (productivity), species composition and relative abundance (diversity). Three management processes are having major impacts upon botanical changes:

- Mowing effect - in April 1998 fencing was erected around the site, to protect it from all grazing animals, and a mechanical system of cutting (mowing) was implemented. The key change amongst the dominant plant species on the site is the promotion of *Festuca* spp. primarily at the expense of *Agrostis* spp., which appear to be comparatively intolerant of mowing. Stress tolerant plant species have expanded in unfertilised plots where, in contrast to those plots treated with nitrogen and/or lime, there is likely to be a gradual but on-going fall in available soil nutrients as grass clippings are deposited off-site. It is also likely that regular mowing is contributing to the expansion in bryophytes through the maintenance of a sward height of some 6cm.
- Fertilisation effect – productivity is significantly higher within those plots treated with nitrogen and/or lime although there are signs that productivity may have peaked in those plots treated with both nitrogen and lime where mean soil pH is now close to 7.0. Distinct differences in the functional structure of plant communities are apparent within lime-treated plots compared to all other treatments, probably resulting from the extremely high levels of lime applied (equivalent to 6 tonnes ha<sup>-1</sup> yr<sup>-1</sup>).
- Insecticide effect – plant species diversity, as measured by the Shannon Diversity Index, is highest within the insecticide-treated plots. Whilst it is recognised that the evidence is only circumstantial at this stage, the possible impact of the soil insecticide, Dursban 4 (Dow AgroSciences) upon plant diversity is discussed. In particular, consideration is given to the possible role of reduced plant herbivory.

# CONTENTS

	Page
<b>1. Introduction</b>	<b>1</b>
<b>2. Methods</b>	<b>4</b>
2.1 Site management and application of treatments	4
2.2 Site data collection	4
2.3 Research group site activity	6
<b>3. Results</b>	<b>7</b>
3.1 Automatic weather station	7
3.2 Above-ground biomass estimates	7
3.3 The interaction between soil pH, moisture content and above-ground productivity	10
3.4 Point Analysis survey	11
<b>4. Discussion</b>	<b>21</b>
4.1 Mowing effect	21
4.2 Fertilisation effect	22
4.3 Insecticide effect	23
4.4 Trampling effect	24
<b>5. References</b>	<b>25</b>
<b>6. Appendices</b>	
1 Soil Biodiversity site plan	27
2 Summary of site- and plot-level treatments	28
3 Species list	29
4 Summary of site activity	30
5 Automatic weather station data	31
6 Above-ground biomass per plot	32
7 Rank of above-ground biomass samples per plot	33
8 Percentage rank abundance of species per treatment	34

## INTRODUCTION

It is now four years since the establishment of the NERC Soil Biodiversity Thematic Programme, centred upon the intensive study of a large field experiment located at the Macaulay Land Use Research Institute's farm at Sourhope in the Scottish Borders. The primary aims of the Programme are to achieve simultaneously an understanding of the biological diversity of the soil biota and the functional roles played by soil organisms in key ecological processes. In seeking to achieve these aims 24 separate research projects have been funded to study soil structure, soil processes (such as the carbon and nitrogen cycles) and the roles of micro-fauna and flora (Bacteria, Nematoda, Protozoa and Fungi), microarthropods (including Collembola and Acari), invertebrate root feeders (Tipulid, Bidionid and Scarabeid larvae), meso-fauna (such as Enchytraeidae) and macro-fauna (including Megradili, Mollusca and Coleoptera). Sixteen of these projects were funded under Phase I (1998-2001) whilst the remaining eight projects have been funded under Phase II. Full details of the each project are available on the Soil Biodiversity website <http://www.nerc-merlewood.ac.uk/soilbio/index.html> or from the Centre for Ecology and Hydrology, Windermere Road, Grange-over-Sands, Cumbria LA11 6JU (Tel 01539 532264).

It is understandable that funded projects under the Soil Biodiversity Programme deal almost exclusively with below-ground activity. It was, nevertheless, recognised at the outset that there are close links to the above-ground vascular and non-vascular plant communities, and feedback mechanisms which are likely to result in concomitant changes within both systems. A botanical survey of the site, which was undertaken in 1998 prior to the start of the Programme, found that the site equated to NVC community U4d consisting of "*Festuca ovina-Agrostis capillaris-Galium saxatile* grassland, *Luzula multiflora-Rhytidiadelphus loreus* subcommunity" (Kenny, 1998). Following this, broad scales of botanical monitoring have been undertaken as part of the site management with the work focussing, in particular, upon above-ground biomass production (productivity), species composition and relative abundance (diversity).



The site was grazed by sheep until April 1998, when fencing was erected to deny access to all grazing animals including deer and rabbits. In 1999 a management regime was put in place to carry out five summer cuts to 6cm every four weeks beginning in May each year. In addition there are five prescribed treatments which can essentially be separated between those plots to which nitrogen and/or lime are added (fertilised) and those to which nitrogen and/or lime have not been added (unfertilised). In broad terms it is expected that productivity in the fertilised plots will increase, but remain level or perhaps, even, fall in the unfertilised plots due to the loss of nutrients contained in the grass cuttings which are deposited off-site

The impact upon plant species diversity is less certain. In their study of *Agrostis/Festuca* plant communities under different sheep grazing management regimes at two sites in Scotland, Hulme *et al.* (1999) report that changes in botanical composition were small and few species invaded or were lost when grazing stock were excluded. However, in a separate study of a range of plant communities following cessation of burning and the reduction or elimination of grazing, Ball (1974) reports that changes in structure and floristics were least marked in species rich *Agrostis/Festuca* grassland and most marked in species poor *Agrostis/Festuca* grassland, with the number of species in ungrazed plots falling by about a half over 12 years. In contrast to these studies, however, the Soil Biodiversity site has been mechanically cut since grazing stopped.

Under the fertile treatments the impact of mowing is unlikely to have any particularly adverse effect upon those species which are able to make use of the greater availability of nutrients, since these species are likely to be more competitive with faster re-growth following cuts. Within these treatments it, therefore, seems likely that plant communities will become less diverse as a few plant species become dominant, displacing often slower growing and smaller species. Within the unfertilised plots growing conditions are likely to become less favourable over time, as nutrients are lost from the system in the grass cuttings, with the possible loss of certain species such as *Festuca rubra* and *Poa pratensis*, which are closely associated with improved pastures. It is likely, however, that this would be a long-term consequence and, in the shorter-term diversity is likely to

remain stable or perhaps increase a little as more nutrient-demanding species contract allowing other species to expand or establish in their place.

Whilst changes in species richness and diversity are of interest, it is perhaps of more relevance to the various Programme Research Groups to know whether there have been any changes to the functional nature of the plant communities. In seeking to answer this question it is important to recognise that plants may be separated into functional groupings or types using very different criteria. Population biologists, for example, seek to define functional types through the measurement of demographic patterns, particularly relating to the spatial and temporal distribution of reproduction, mortality, dispersal and dormancy (eg Harper 1977; Whittaker & Goodman, 1979). In contrast, physiologists define functional types by reference to those features of life history, physiology and biochemistry that determine the responsiveness of plants to soils, land-use and climatic factors (Grime *et al.*, 1996). These have resulted in the identification of functional types based upon factors such as differences in phenology (eg Raunkiaer, 1934; Al-Mufti *et al.*, 1977) and plant species' distribution relative to water supply (eg Ellenberg, 1988). The CSR system (Grime, 1979) is a comprehensive functional classification of common British vascular plants, which uses a mix of demographic and physiological information. This system identifies three permutations of environmental extremes and concludes that those species typically associated with these extremes all possess distinct sets of traits leading to characteristic ecological behaviour. In the case of low stress and low disturbance this is competitiveness (C), in the case of high stress and low disturbance this is stress-tolerance (S) and in the case of low stress and high disturbance this is ruderality (R). The fourth environmental contingency, that of high stress and high disturbance, does not support plant life. The initials of the three "primary" types give the CSR system its name. Intermediate types also exist within the system, with each representing a different intermediate combination of stress and disturbance.

Nutrient stress is likely to be reduced within the fertilised plots suggesting that there will be a functional shift from stress tolerators towards more competitive plant species. Within the unfertilised plots the removal of disturbance and spot fertilising, arising from

the presence of grazing livestock, is replaced by the more uniform disturbance of mowing coupled with a gradual reduction in fertility as nutrients are lost in clippings which are deposited off-site. In these circumstances stress tolerant species are likely to be the biggest beneficiaries.

## **METHODS (INCORPORATING SITE ACTIVITY)**

The Rigg Foot Experimental Site at Sourhope consists of 5 replicate blocks in downslope environmental gradients. Each block contains 6 main plots with treatments allocated at random. The main plots are subdivided into 10 sub-plots and areas are allocated to different research groups using a 0.5m x 0.5m cell system. Appendix 1 provides a map of the site, showing treatments allocated to each plot.

### *2.1 Site management and application of treatments.*

There are one site-level and three plot-level management tasks, which have been undertaken each year since 1999. Vegetation across the whole site is mowed monthly between May and September to a level of approximately 6cm using a Kubota riding grass mower. Immediately following these cuts Dursban 4 OPA insecticide (ex Dow AgroSciences), containing 44.6% chlorpyrifos w/w, is applied to the Insecticide plots (previously referred to as Biocide plots). Nitrogen and/or lime is added to designated plots during spring. Full details of the dates and, where appropriate, the application rates of these treatments are provided in Appendix 2.

### *2.2 Site data collection.*

- Regular temperature, rainfall and other weather data have been collected since February 1999 from an on-site Automatic Weather Station.
- Estimates of above-ground productivity involve the collection of vegetation samples from a randomly chosen 0.5m<sup>2</sup> cell within sub-plots S, T, U & V of every plot

immediately prior to each mow. The clippings are oven-dried at 80°C and subsequently weighed. Samples have been collected since 1999.

- Between 27<sup>th</sup> July and 7<sup>th</sup> August 1998 a baseline assessment of vegetation was undertaken using a 0.5m<sup>2</sup> point quadrat consisting of 25 points. In this survey five randomly selected sub-plots were surveyed in each main plot. Each point of the quadrat was followed down to the soil surface where a record was made of the single species which occurred at this precise point.

More extensive botanical surveys, using the point analysis technique, have subsequently been undertaken in July/August in the years 2000, 2001 and 2002. In these surveys a separate 0.5m<sup>2</sup> cell within each of sub-plots S, T, U & V has been used each year. A point quadrat frame, containing a grid of one hundred holes, is placed over the appropriate cell. A large pin is dropped through the same twenty five holes each time and a record made of each occasion a species is touched by the pin down the vegetation profile.

During the 2002 point analysis survey an effort was made to identify bryophytes to species' levels in contrast to previous years when they were only separated to *Rhytidiadelphus squarrosus*, other mosses and liverworts. Appendix 3 contains a full species list together with details of other species seen elsewhere on the site.

- Soil samples have been collected regularly since 1998 to monitor soil pH (measured in H<sub>2</sub>O) at approximately 5cm down the soil profile. During 2002 soil pH was measured in the centre of each plot in March and in the centre of each cell used in the botanical point analysis survey in July/August.
- On 16<sup>th</sup> August 2002 a theta probe was used to measure soil moisture content in each corner of every cell used in the botanical point analysis survey. These measures have not been taken as a specific management task in previous years, although it is recognised that some research groups regularly collect soil moisture measurements as part of their studies.

- Additional small-scale botanical surveys have been undertaken at various times:
  - vegetation cover estimates within nine separate 5cm x 5cm squares (0.0225m<sup>2</sup>) above buried minirhizotron tubes in Control 1, Insecticide and Nitrogen & Lime plots (2000, 2001 & 2002). Project 2113.
  - Biomass samples whereby vegetation was cut to ground level, sorted to species, dried and weighed. Year 2000 (excl. C2) samples 250mm x 250mm (0.0625m<sup>2</sup>). Year 2002 (C2, Insecticide, Nitrogen only and Lime only, blocks 1,2 & 3 only) samples 250mm diameter circles (0.049m<sup>2</sup>). Projects 2109 & 2115 respectively.
  - vegetation cover estimates in sub-plot Y disturbance:fertility gradients within C1 plots only. Year 2000 (all – 6.25m<sup>2</sup>) and 2001 (half – 3.25m<sup>2</sup>). Project 2133.

Full details of these surveys may be obtained from the Project PI's.

### *2.3 Research group site activity.*

Fieldwork associated with the sixteen projects funded under Phase I has largely finished, although there is still some occasional activity primarily from groups collecting samples and/or additional data to conduct further small-scale studies. Researchers from seven of the eight projects funded under Phase II have visited Sourhope during 2002 to undertake major fieldwork activities. Many of these have involved labelled <sup>13</sup>CO<sub>2</sub> pulses and/or the measurement of trace gases using two mobile laboratories from the Centre for Ecology and Hydrology (CEH), which were based on the Soil Biodiversity site for some 6 weeks at various times during June, July and September.

Research group activity on the site reverted to more normal levels, following the reduction in 2001 when restrictions upon site visits were imposed following the outbreak of foot and mouth disease (Appendix 4). Soil samples collected from the site since the start of the experiment now total 11621.

## RESULTS

### 3.1 Automatic Weather Station.

Appendix 5 provides headline weather data at the site since the start of the experiment. When compared to previous records the main points of note in 2002 were low rainfall in April and September offset by wet periods in February, July and the last 3 months of the year resulting in rather higher than average rainfall for the year. In temperature terms Winter 2001/02 was extremely mild, with an average air temperature of  $3.88^{\circ}\text{C}$  for the months of December to February compared to  $2.28^{\circ}\text{C}$  and  $2.86^{\circ}\text{C}$  for the same periods in 2000/01 and 1999/2000 respectively.

### 3.2 Above-ground biomass estimates.

As in previous years, there was a consistent hierarchy throughout the summer of biomass samples collected from plots subjected to the different treatments (Figure 1). Unlike

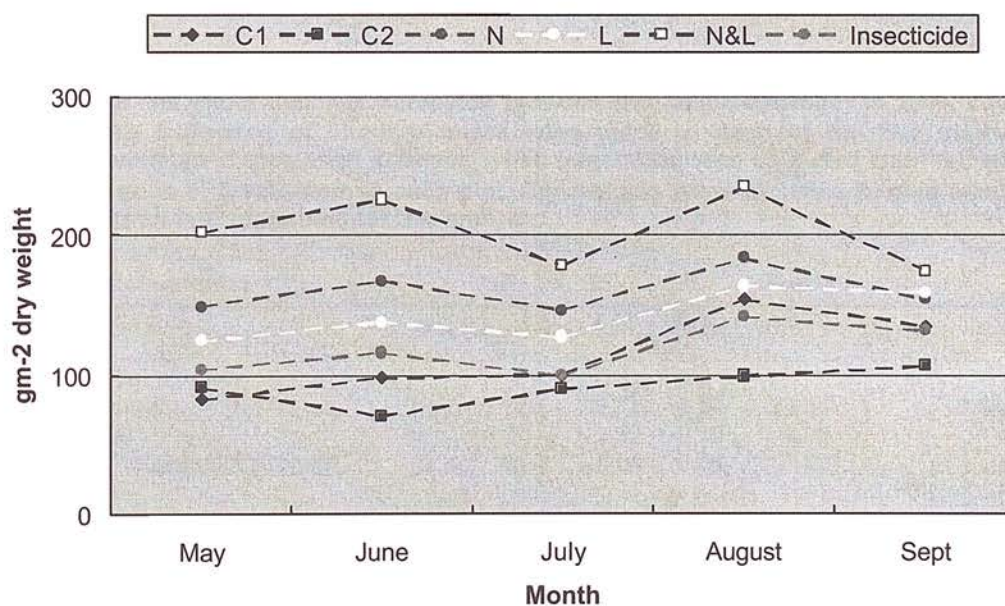


Figure 1 - Analysis of treatment differences between monthly above-ground biomass harvests (May-Sept 2002)



previous years, however, production in mid-summer fell across a number of treatments. Biomass samples collected from the Control 2 plots, which were used by research groups for sampling for the first time during 2002, often followed a different monthly pattern to that of the other treatments. In absolute terms biomass productivity fell in 2002 compared to 2001, but this does not deter from the longer-term trend of increasing biomass production across all treatments compared to their starting point in 1999 (Figure 2a). The positive impact of nitrogen and, to a lesser extent, lime upon productivity is clearly visible in Figure 2 (b), in which biomass samples from each treatment have been normalised against the corresponding Control 1 samples in each year. The most effective promotion of above-ground biomass continues to be seen within the plots in which nitrogen and lime have been applied together, whilst the Control 2 plots were less productive than the Control 1 plots for the third time in four years. Whilst productivity within the most fertile nitrogen and lime plots has marginally increased relative to the Control 1 plots in 2002 compared to 2001, it is apparent that all other treatments have reduced. Split-plot ANOVA's, using ln-transformed biomass dry weights, reveal statistically significant differences between a number of treatments in 2002 (Figure 2c) and a significant interaction between treatment and year (Table 1).

**Table 1 Results of split plot ANOVA (Genstat 6) examining the effects of Treatment, Year and Interaction on shoot biomass harvested between May and September in 1999, 2000, 2001 and 2002. Estimates of shoot biomass were made at each of the five mowing occasions conducted during each summer, when vegetation was collected from random 0.5m<sup>2</sup> cells in each of 4 sub-plots in each plot. Dry weights were summed, to give annual biomass, and then ln-transformed for this analysis.**

Source of variation	df	SS	MS	F-ratio	P
Block stratum	4	0.76	0.19	1.30	
Block.Plot stratum					
<b>Treatment</b>	5	20.72	4.14	28.38	<0.001
Residual	20	2.92	0.15	3.25	
Block.Plot.Subplot stratum	90	4.04	0.04	1.65	
Block.Plot.Subplot "Units" stratum					
<b>Year</b>	3	46.36	15.45	565.99	<0.001
<b>Treatment.Year</b>	15	2.00	0.13	4.89	<0.001
Residual	342	9.33	0.03		
Total	479	86.15			

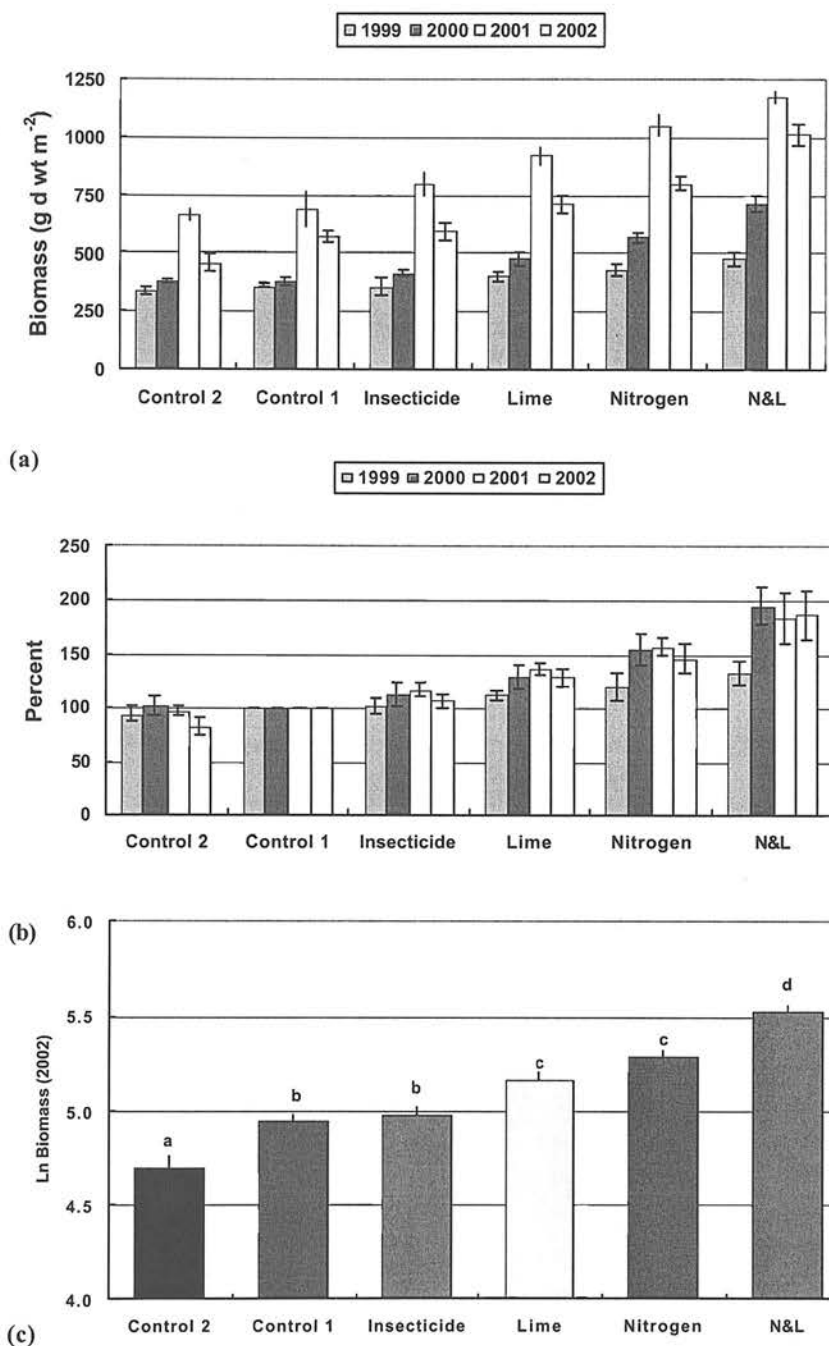


Figure 2 – (a) Above-ground biomass, summed from harvests made at each of the five cuts each summer from 1999 to 2002. (b) – Treatment biomass samples expressed as a percentage of Control 1 estimates in each year. (c) – Results of split-plot ANOVA (Genstat 6) showing effect of treatment on shoot biomass in summer 2002 (data ln transformed  $F=38.97$   $df=4,5,20,90$   $p<0.001$ ). Means with the same letter do not differ significantly (Isd test  $p>0.05$ ).

Appendix 6 shows total biomass sampled within each plot since 1999, together with the mean within each block during the same period. Sampled biomass reduced across all treatments in 2002 compared to 2001. Reductions ranged from 13.7% in the N&L plots up to 31.2% in the Control 2 plots. Although there were no significant differences between blocks (ANOVA,  $F=0.34$   $df=5,4,20$   $p>0.05$ ) there was a clear negative relationship between location on the slope and productivity, with Block 5 recording a fall of 16% in sampled biomass whilst Block 1, at the bottom of the slope, recorded a fall of 30%. A similar pattern was also evident in 2001 when large increases in sampled biomass weights were observed across the site compared to 2000. On this occasion the lowest percentage increase was seen in Block 1.

Appendix 7 shows total estimates of annual biomass productivity within each plot in 2002 together with cumulative totals from 1999 to 2002.

### 3.3 The interaction between soil pH, moisture content and above-ground productivity.

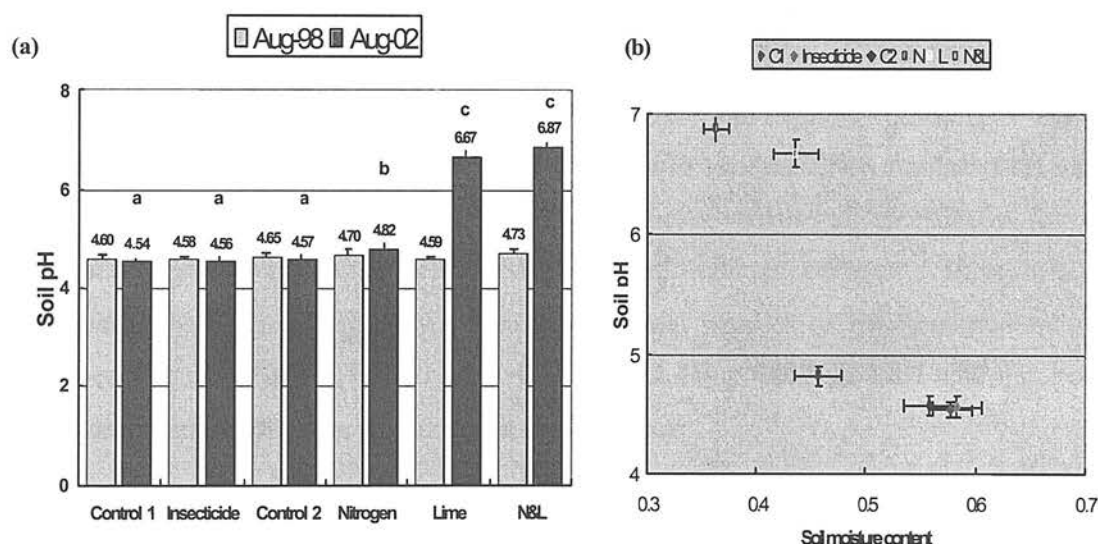


Figure 3 – (a) Changes in soil pH (5cm depth, measured in distilled water) across different treatments in August 2002 compared to baseline samples taken from the same plots prior to the start of the experiment in August 1998, at which time means were not significantly different. Letters are used to indicate treatments with significantly different means in 2002. Test by LSD after general ANOVA ( $F=230.79$   $df=4,5,110$   $p<0.001$ ). Four samples were collected from each plot during August 2002. Bars show  $\pm$  one standard error. (b) - Link between Soil pH, soil moisture content and treatments as measured in July/August 2002

Figure 3a shows that the addition of lime has had a highly significant positive effect on soil pH, at least in the upper section of the soil profile, with the result that average soil pH is now approaching seven within both limed treatments. Nitrogen has had a smaller, but still statistically significant positive impact whilst the controls and Insecticide treated plots are virtually unchanged from their 1998 starting points. There is a strong negative correlation between soil moisture content and soil pH (correlation coefficient,  $r=0.68$ ,  $n=120$ ) with a clear separation between the improved plots and the wetter, more acidic unimproved plots (Figure 3b).

Figure 4a shows strong positive linear ( $r=0.69$ ) and polynomial ( $r=0.73$ ) correlations between upper soil profile pH and biomass productivity when all treatments are included. However, if each treatment is considered in isolation (Figure 4b) this positive correlation becomes less significant within those plots to which nitrogen or lime have been added, whilst the relationship actually becomes negative in those plots to which both nitrogen and lime have been added, suggesting the polynomial relationship is more accurate.

### 3.4 Point Analysis survey

The Point Analysis survey in July/August 2002 was conducted by Graham Burt-Smith together with Willow Walker, a botanical surveyor employed specifically for this purpose. Each was responsible for 50% of all quadrats surveyed. There was no statistically significant difference between the total number of hits recorded by each surveyor (general blocked ANOVA  $F=0.31$ ,  $df=4,1,114$   $p>0.05$ ) and the total number of hits recorded in 2002 was similar to the previous year.

Appendix 8 reveals clear treatment differences in the frequency with which species were encountered in the 2002 point analysis survey. In particular, two grass species commonly associated with improved pastures (*Festuca rubra* and *Poa pratensis*) were relatively abundant within the improved plots, accounting for 53% of total hits within the Nitrogen and Lime-treated plots, 30% within the Lime-treated plots and 25% within the Nitrogen-treated plots, but failed to account for more than 10% of total hits within any of the

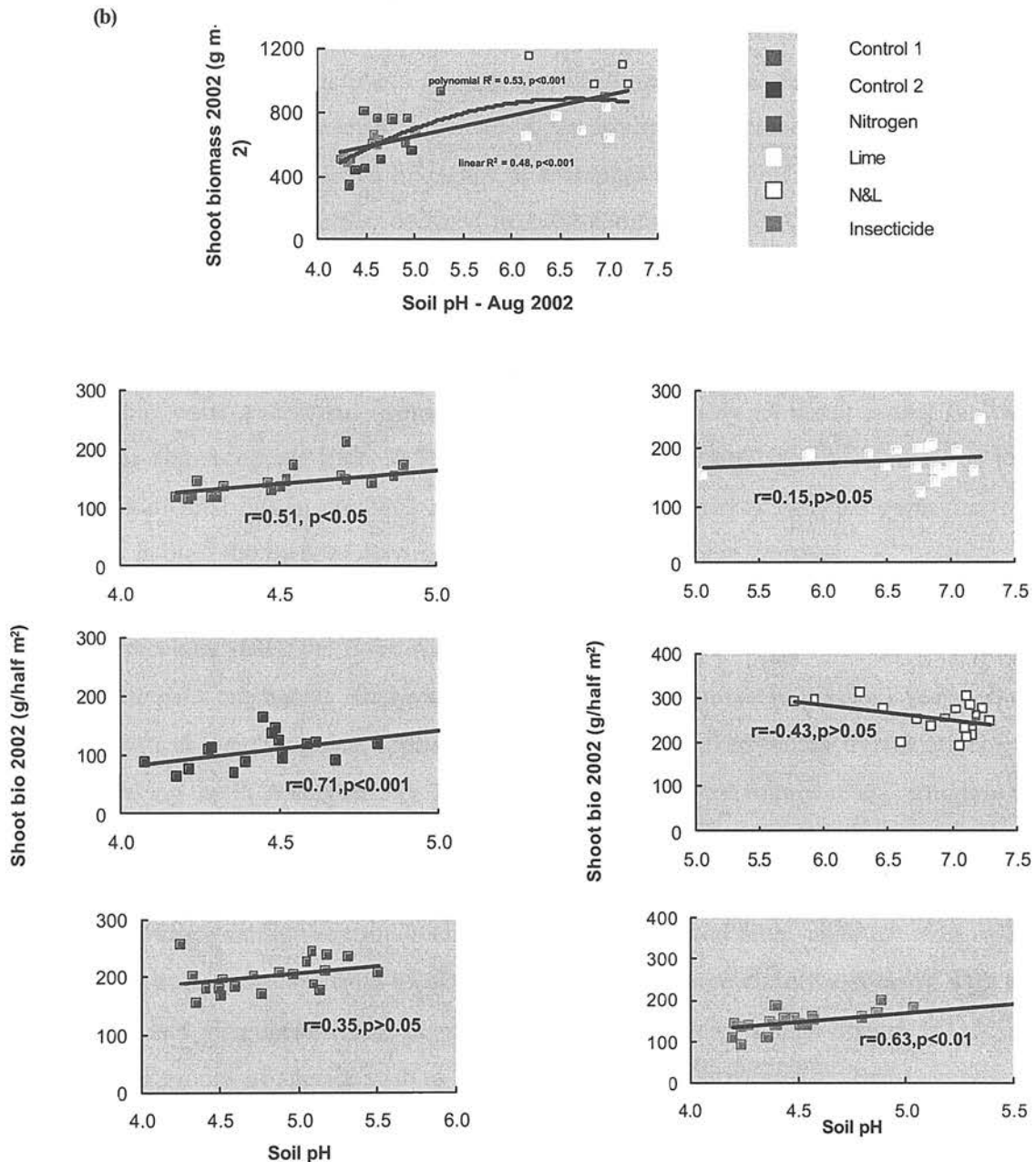


Figure 4 – Above-ground biomass sampled during summer 2002 plotted against soil pH measured during August 2002. (a) All plots (per plot). (b) Within treatments (4 samples per plot).

Control or Insecticide-treated plots. In contrast two grass species commonly associated with unimproved pastures (*Festuca ovina* and *Anthoxanthum odoratum*) accounted for just 9% of total hits within the Nitrogen and Lime treated plots whilst the total percentage

of hits in the other treatments ranged from 16% within the limed plots up to 30% within the Control 2 plots. *Agrostis* and *Festuca* spp. continue to be the dominant vascular plant species at the whole-site level. Although *Agrostis capillaris* remains the most abundant individual species the relative frequency of encounter with each of the two *Agrostis* spp. (both *capillaris* and *vinealis*) declined in 2002 compared to 2001 continuing the trend first noted in last year's Annual Report. In contrast a relative fall in *Festuca ovina* "hits" (down 25% in 2002 compared to 2001) was accompanied by a rise in *Festuca rubra* "hits" (up 43% over the same period), possibly reflecting identification difficulties in separating weak *rubra* and strong *ovina* specimens. The overall result is that the two *Festuca* spp. accounted for 30.76% whilst the two *Agrostis* spp. accounted for just 25.32% of total hits in the 2002 survey. This contrasts sharply with the corresponding figures in the 2000 survey (*Agrostis* spp. 43.28%, *Festuca* spp. 23.26%).

As in previous years, the Point Analysis data from the 2002 survey have been subjected to Principal Components Analysis (PCA). However, in contrast to previous years, when no statistical weighting was applied, species were first ranked within treatments before undertaking the PCA (Figure 5a). The results broadly separate improved and unimproved plots. In particular there is a clear grouping of those plots treated with lime. There is evidence that a number of nitrogen-treated samples, particularly from blocks one, two and five, share similar vegetation characteristics to the limed plots, although most of the nitrogen-treated samples from blocks three and four are more closely associated with the controls and insecticide-treated plots. A plot of latent vector loadings (Figure 5b) clearly separates groups of species such as *Poa pratensis*, *Festuca rubra* and *Trifolium repens*, which are commonly associated with more fertile growing conditions, from others such as *Festuca ovina*, *Agrostis vinealis*, *Luzula multiflora* and *Anthoxanthum odoratum*, which are usually associated with less fertile growing conditions.

Appendix 3 shows that six separate mosses and one liverwort were identified on the plots during surveys in 2002. Bryophytes, in particular *Rhytidiadelphus squarrosus*, continue to become more abundant across the whole site and account for 19.92% of total hits in



the 2002 survey compared to 10.82% in 2001 and 6.48% in 2000 (Appendix 8). Split-plot ANOVA reveals a highly significant difference between treatments in the frequency with

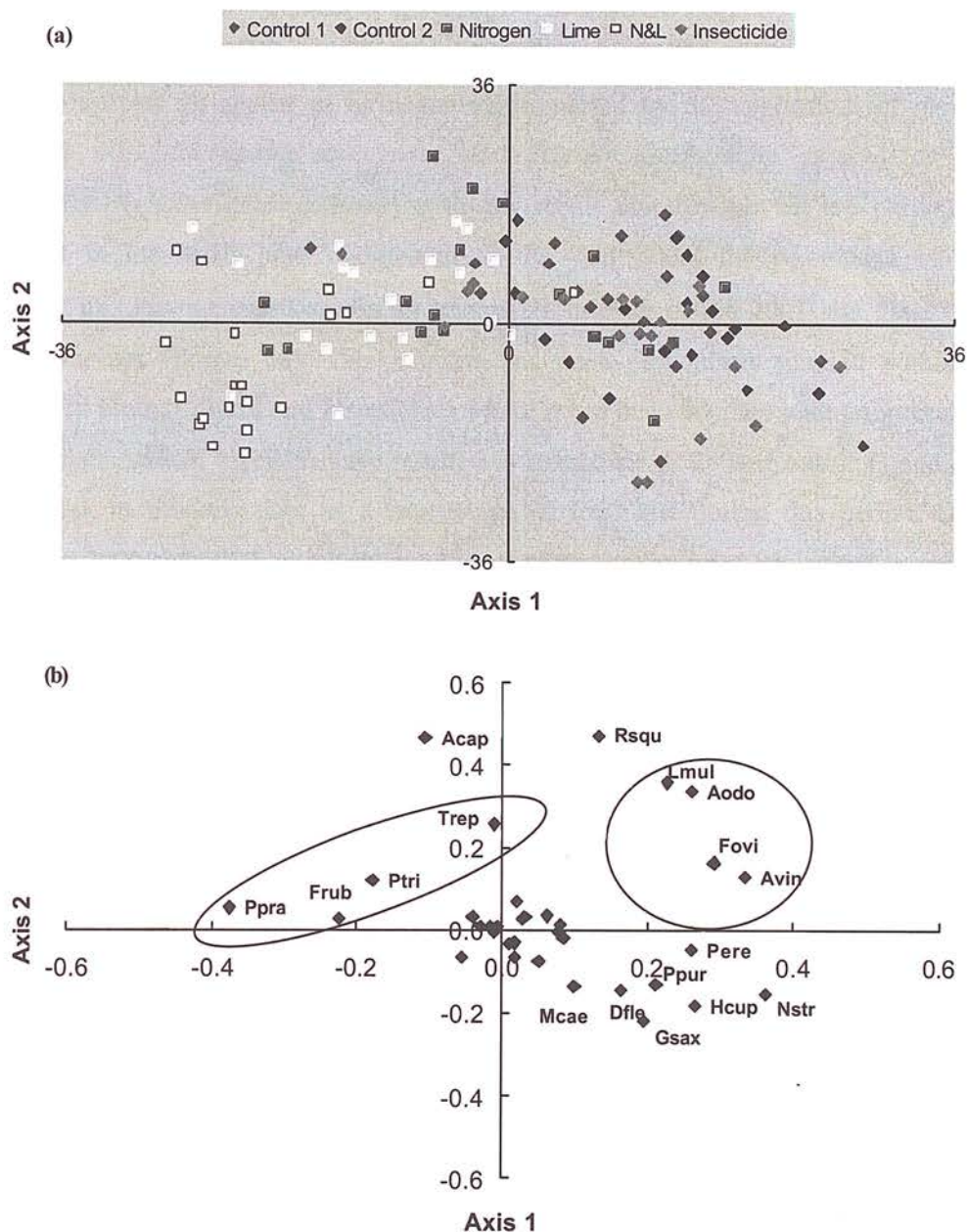


Figure 5 – A Principal Components Analysis (PCA - Genstat 6) has been undertaken using data from the 2002 point analysis survey. Species were first ranked according to number of hits in each sample. Percentage variation explained by axis 1 is 24.4% and axis 2 is 11.1%. (a) PCA scores. (b) PCA latent vector loadings for each of the 34 vascular plant and bryophyte species recorded in the survey

which bryophytes are encountered ( $F=6.43$   $df=4,5,20,90$   $p<0.001$ ). At the species level, the relative abundance of *Eurynchium praelongum* is similar in each treatment but other bryophyte species all appear to be negatively impacted by the application of nitrogen (especially *Rhytidiadelphus squarrosus* and *Pseudoscleropodium purum*) or lime (particularly *Hypnum cupressiforme*) with the result that bryophytes are particularly infrequent in the N&L plots. Compared to the year 2000, the percentage of total bryophyte hits has increased across all treatments in each of the 2001 and 2002 point analysis surveys (Figure 6a). This increase has been particularly marked within the unimproved plots (Control and Insecticide) where it has been coupled with an increase in dicot numbers which, together, have resulted in reductions of 24% (Control 1) and 18% (Insecticide) in monocot hits as a percentage of total hits during this period. Other surveys, in 2000 and 2002, in which small areas of vegetation were cut, sorted to species, dried and subsequently weighed reveal an even more dramatic increase in the contribution of bryophytes to the overall biomass in the unimproved plots (Figure 6b).

In 2000 there were no significant differences between treatments in the frequency with which dead vegetation (litter) was encountered in the point analysis survey (ANOVA  $F=0.13$ ,  $df=4,4,16$   $p>0.05$ ). Figure 7, however, shows that statistically significant differences between the various treatments have emerged in 2001 and 2002, with the N&L plots recording the highest mean number of hits whilst the Control 1 plots have recorded the lowest.

Shannon Diversity Indices have been calculated using the basic point analysis data from the initial baseline data survey in 1998 together with the subsequent more extensive point analysis surveys in 2000, 2001 and 2002 (Figure 8). Prior to the start of the experiment there were no statistically significant differences across the site (split-plot ANOVA,  $F=0.49$   $df=4,5,20,120$   $p>0.05$ ). Evidence of treatment differences became apparent in 2000 (split-plot ANOVA,  $F=3.72$   $df=4,4,16,75$   $p<0.05$ ) and became more pronounced in both 2001 (split-plot ANOVA,  $F=10.60$   $df=4,4,16,75$   $p<0.001$ ) and 2002 (split-plot

ANOVA,  $F=17.91$   $df=4,5,20,90$   $p<0.001$ ). When compared to the baseline data survey, the 2002 Shannon Diversity Index has fallen by 15% in the nitrogen and lime treated plots in contrast to all the other treatments which have seen increases in the Shannon Diversity Index ranging from a minimum of 2% in the limed plots up to a maximum of 29% in the Insecticide-treated plots.

There are three predominant functional groups - stress tolerant (S), C-S-R and SR/C-S-R species – which have, together, accounted for a minimum of 69% and a maximum of 95% of total vascular plant hits in any one plot in the various Point Analysis surveys which have been undertaken (Table 2).

**Table 2 – Percentage of Point Analysis survey live vascular plant “hits” represented by stress tolerant (S), C-S-R and SR/C-S-R plant species prior to the start of the treatments (1998) and in subsequent years.**

	Minimum %	Maximum %
1998	69	95
2000	75	99
2001	76	99
2002	80	99

Within each of these functional groupings there were no significant differences between the plots allocated to each treatment prior to the start of the experiment in 1998 (Figure 9a). However, treatment differences within the SR/C-S-R functional group emerged in 2000 and, by 2002, each of the three functional groupings recorded highly significant statistical differences between treatments (Figure 9b-d). C-S-R species have greatly expanded to become dominant within the nitrogen and lime plots, whilst stress tolerant species are particularly benefiting within the unimproved plots, although they are also showing modest gains within those plots treated with nitrogen-only and lime-only. SR/C-S-R species are particularly reduced within the lime- (with and without nitrogen) and insecticide -treated plots.

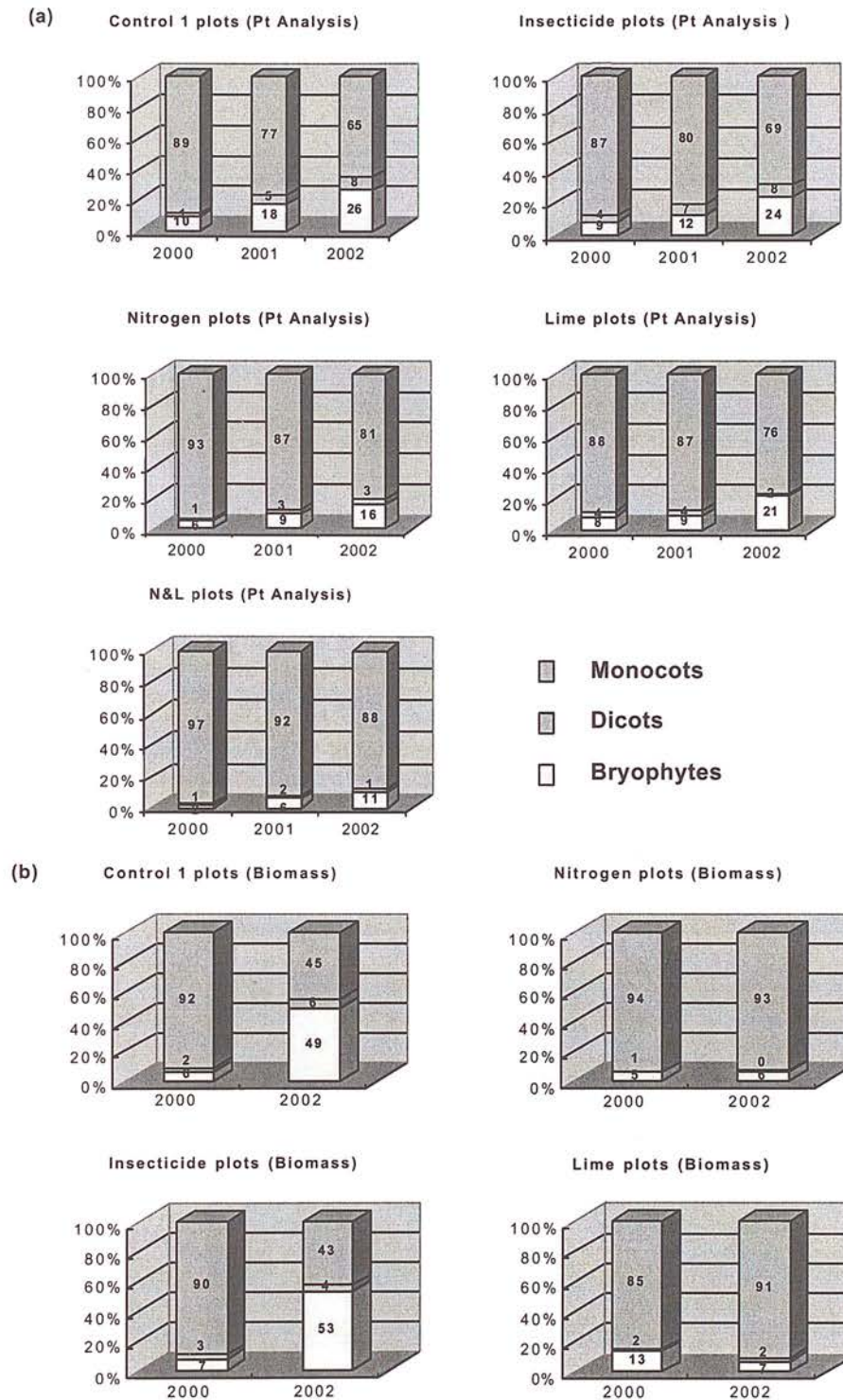


Figure 6 – Changes in fundamental botanical groupings within different treatments analysed from various surveys during 2000 to 2002. (a) Point analysis surveys – frequency within each group is expressed as a percentage of the total for that particular year. (b) Biomass surveys in 2000 ( $n=5$ , sample size  $6250\text{mm}^2$ ) & 2002 ( $n=3$ , sample size  $4900\text{mm}^2$ ).



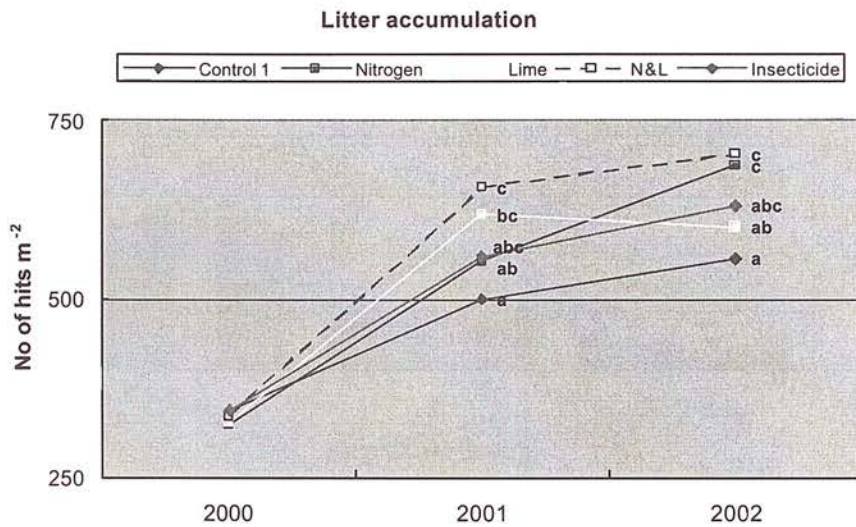


Figure 7 – The mean number of litter hits  $\text{m}^{-2}$  within each treatment from the point analysis surveys in each of the past 3 years (error bars omitted to aid clarity). ANOVA's have been undertaken for each year: 2000  $F=0.13$ ,  $df=4,4,16,75$   $p>0.05$  2001  $F=3.46$   $df=4,4,16,75$   $p<0.05$  2002  $F=3.52$   $df=4,5,20,90$   $p<0.05$ . In 2000 there were no statistically significant differences between means. Thereafter means with the same letter do not differ significantly within years (Isd test  $p>0.05$ ). Control 2 plots are excluded.

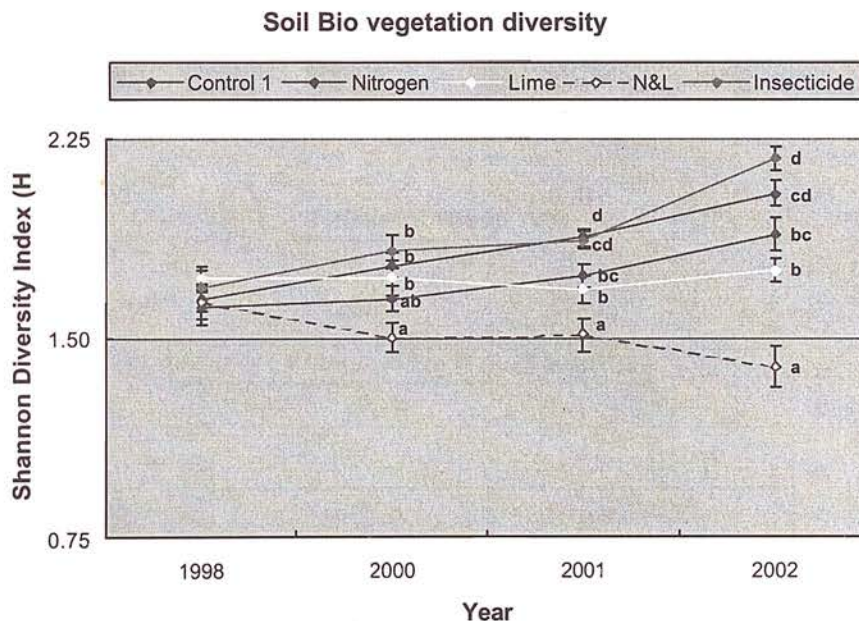
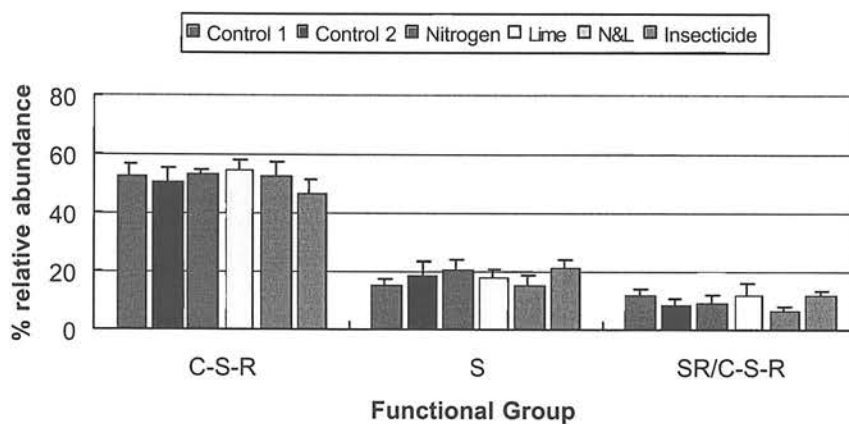


Figure 8 - Mean Shannon diversity indices have been calculated for each treatment using data from the point analysis surveys. Standard error bars are shown. In 1998 there were no statistically significant differences between means. Thereafter means with the same letter do not differ significantly within years (Isd test  $p>0.05$ )

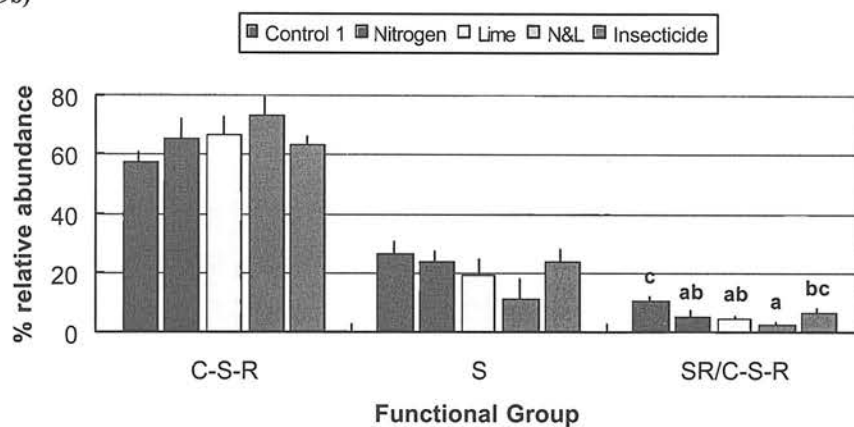
(9a)

1998



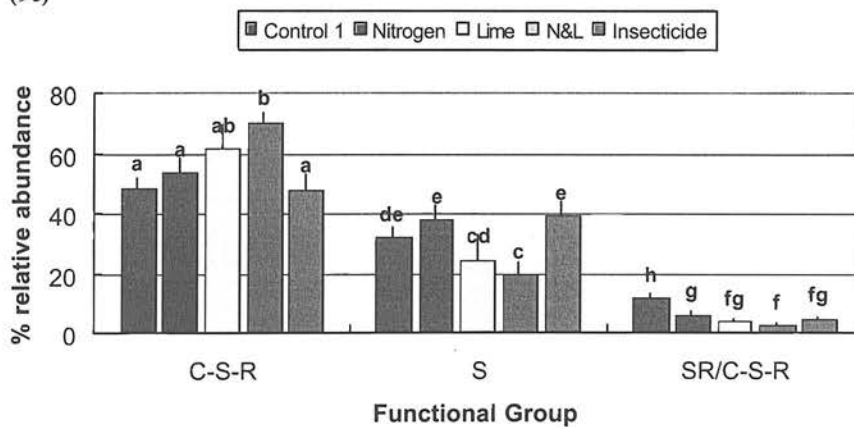
(9b)

2000



(9c)

2001





(9d)

2002

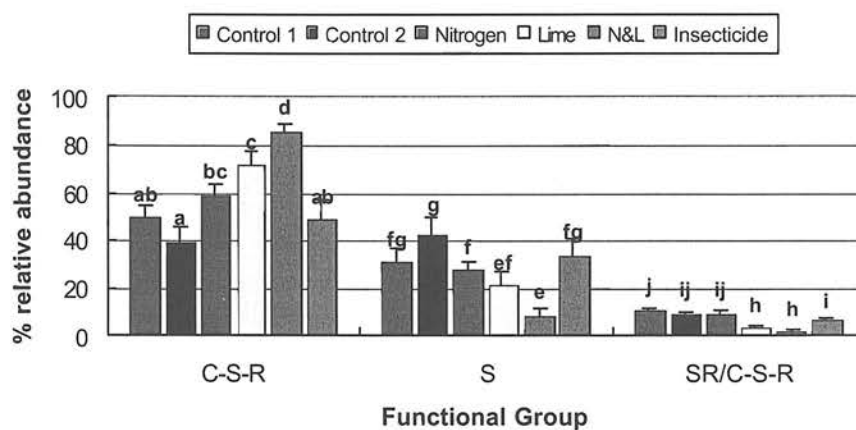


Figure 9 – Relative abundance of key functional groups of vascular plant species shown as percentage of total live vascular plant “hits” in point analysis surveys. S=stress tolerators, CSR=competitive stress ruderals and SR/CSR=stress ruderal/competitive stress ruderals (Grime, 1977). Means with the same letter do not differ significantly (lsd test  $p>0.05$ ). Bars indicate plus one standard error.

ANOVA's:

Fig	CSR	S	SR/CSR
9a	$F=1.25$ $df=4,5,20$ $p>0.05$	$F=0.76$ $df=4,5,20$ $p>0.05$	$F=0.96$ $df=4,5,20$ $p>0.05$
9b	$F=2.03$ $df=4,4,16$ $p>0.05$	$F=2.10$ $df=4,4,16$ $p>0.05$	$F=4.94$ $df=4,4,16$ $p<0.01$
9c	$F=4.29$ $df=4,4,16$ $p<0.05$	$F=5.18$ $df=4,4,16$ $p<0.01$	$F=13.54$ $df=4,4,16$ $p<0.001$
9d	$F=12.86$ $df=4,5,20$ $p<0.001$	$F=6.12$ $df=4,5,20$ $p<0.001$	$F=11.43$ $df=4,5,20$ $p<0.001$

## 4. DISCUSSION

Changes to plant productivity and botanical composition can be summarised under three main headings plus one supplemental heading:

### 4.1 Mowing effect

One of the most striking points to emerge from the Point Analysis surveys is the reduction in both *Agrostis capillaris* and *Agrostis vinealis* which have together declined by over 40% since 2000. This has occurred in approximately equal measures across all treatments, suggesting that the decline in these particular species does not result from the application of any particular treatment but is, rather, due to some site-level factor. One potential explanation is an adverse reaction of these species to the mowing, in particular the homogenous nature of the cuts which do, of course, differ considerably from the heterogeneous grazing of livestock. Other possibilities include climatic or biotic factors such as competitive exclusion by other species. The major vascular plant beneficiary is *Festuca rubra*, which is one of the group of species identified as being associated with plots to which lime and, to a lesser extent, nitrogen have been added. It is unsurprising therefore, that the expansion in relative abundance of this species has been particularly apparent within the more fertile plots.

The expansion of stress tolerant species across all treatments, with the sole exception of nitrogen and lime, suggests that this functional group has also benefited most from some general site-level factor, such as reduced levels of disturbance following the removal of grazing animals. It is suggested that this reduction in disturbance has also been responsible, at least to some extent, for the decline in SR/C-S-R species across all treatments, given the ruderal nature of this functional group of species. Treatment differences across both these functional groups reflect those attributes of stress tolerant species, such as long-living leaves, which enable them to cope more successfully with the lower soil pH and nutrient levels associated with the unimproved treatments.

The widespread expansion of mosses across the site generally supports the study by Hulme *et al.* (1999) who found that maintenance of an *Agrostis/Festuca* grassland sward height at some 4.5cm resulted in a higher content of mosses in the absence of the tussock grass *Nardus stricta*. It is possible that the maintenance of a sward height of approximately 6cm through regular mowing, possibly coupled with the absence of grazing animals for the past five years, has produced conditions enabling a rapid expansion in the relative abundance of bryophytes. This effect is somewhat reduced by the greater vascular plant productivity within the more fertile treatments.

#### *4.2 Fertilisation effect*

Since 1999 there has been an on-going positive effect upon aboveground vegetation biomass (productivity) of soil improvements, involving the addition of nitrogen and/or lime. However, whilst the most productive plots remain those to which both nitrogen and lime have been added, there are some signs that productivity may have peaked within these plots, possibly as a result of their increased soil pH linked with a comparatively low soil moisture content. Whilst it is generally accepted that a Soil pH of around 6.0 provides good growing conditions to optimise agricultural productivity within grasslands, it is apparent that the high annual rates of lime application to designated plots in the Soil Biodiversity project have resulted in their mean soil pH being closer to 7.0 by summer 2002. In graphical terms the relationship between above-ground biomass and soil pH is frequently normally distributed and it is possible therefore that productivity within these plots will continue to decline if soil pH rises again following another application of lime in spring 2003. It is also possible that the extra stimulation to plant growth within those plots to which Nitrogen has been added, in addition to lime, has significantly reduced soil moisture content. This has, in turn, caused the vegetation to be particularly susceptible to water stress during dry periods, probably leading to reduced plant growth. There was some visual evidence of plant stress in summer 2002 when large patches of vegetation suffered from chlorosis, particularly within those plots treated with lime.

The increase in G-S-R species, within those plots treated with both nitrogen and lime, supports the hypothesis that plant species within this functional group have been able to expand by using those attributes, such as growth rate, which enable them to make greater use of the more benign growing environment. For C-S-R species, a higher soil pH appears to be more important than the addition of nitrogen as evidenced by the relatively poor performance of G-S-R species within those plots fertilised with nitrogen only compared to those plots treated with lime only.

#### *4.3 Insecticide effect*

Interactions between plants and herbivores are numerous and extremely complex. Field experiments in grassland plant communities show that animals and birds often, but not always, increase plant diversity whilst plant diversity usually decreases when they are excluded. Studies have demonstrated clear effects of insect herbivory on plant species composition, vegetation cover and structure (Brown, 1982, 1985) although it is apparent that these vary according to the type of insecticide applied and the life history grouping of the plants. For instance, in their study into the differential effects of above- and below-ground insect herbivory during early plant succession Brown and Gange (1989) reported that plant species richness and diversity, in general, were increased by the application of soil insecticide but, by the second season, depressed by foliar insecticide whilst the responses differed between annual herbs, perennial grasses and perennial herbs.

According to the product label, Dursban 4 insecticide (Dow AgroSciences) is effective for controlling the agricultural pests frit fly and leatherjackets on pasture land, although other insects will also be susceptible. However, in the absence of information about the exact nature and levels of insect control, it would be unwise to report a direct link between reduced insect herbivory and a higher Shannon diversity index. It is nevertheless clear that the results mirror those of Brown and Gange (1989) and, if such a link does indeed exist, one possible explanation is that benefits are accruing to a number of more palatable plant species for which conditions are now suitable to survive whereas, previously, they would have been unable to sustain herbivore damage in the relatively

infertile environment at Sourhope. As long as the environment remains relatively infertile these plants should not become dominant and it is likely that plant diversity levels would therefore remain comparatively high.

#### *4.3 Trampling effect*

Sampling within the C2 plots took place for the first time in 2002 and this probably accounts for the dramatic fall in productivity within these plots relative to the C1 plots. In particular, it is likely that vegetation growth in many of the C2 plots was suppressed by trampling during intense periods of activity associated with the application of labelled  $^{13}\text{CO}_2$  pulses on these plots.

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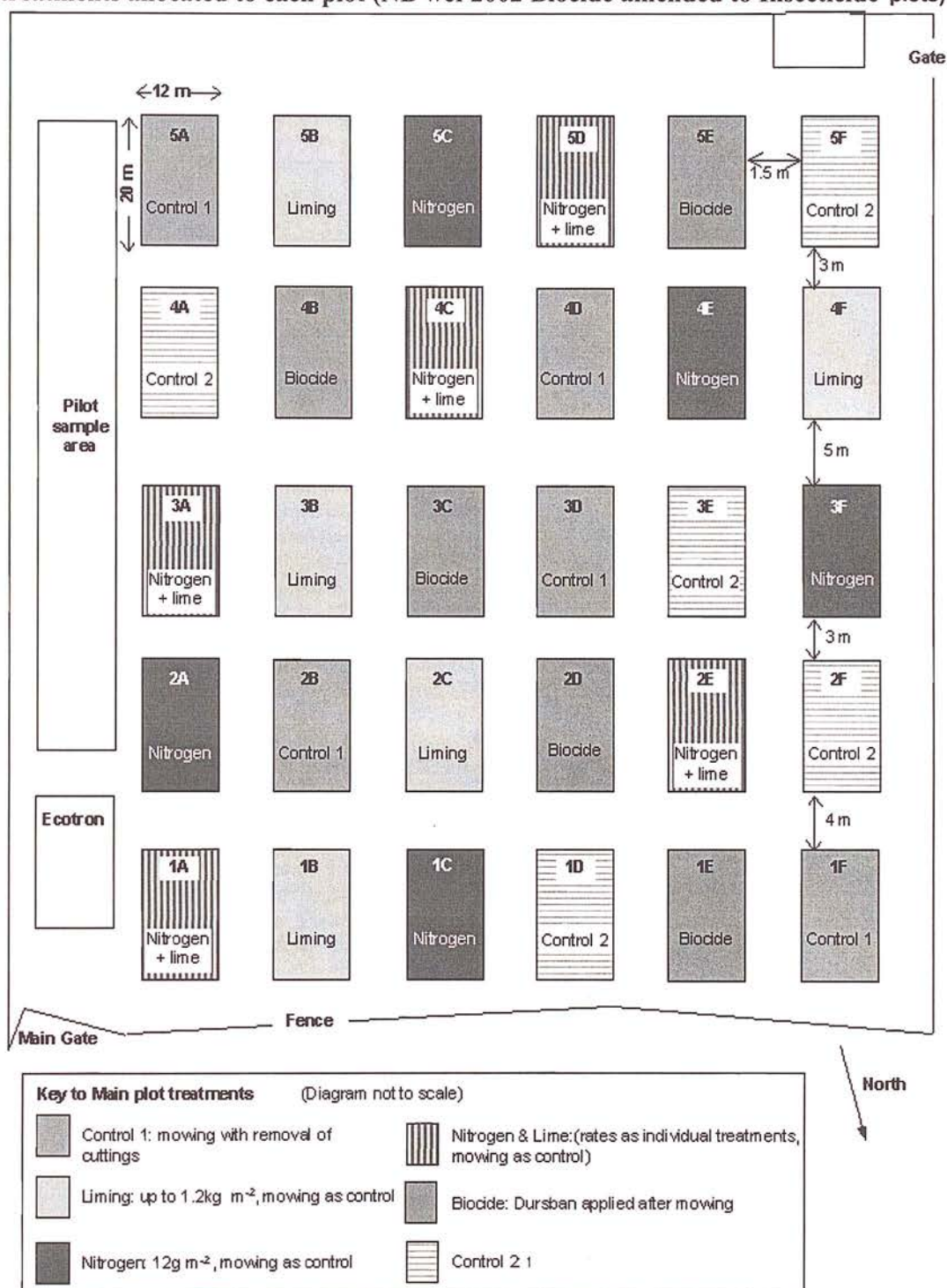
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**Appendix 1 – Soil Biodiversity site, Sourhope. Map showing orientation of site and treatments allocated to each plot (NB wef 2002 Biocide amended to Insecticide plots)**



## Appendix 2 – Summary of Soil Biodiversity site and plot level treatments

### • Site level treatment – vegetation cutting

	1999	2000	2001	2002
Cut 1	1 <sup>st</sup> - 9 <sup>th</sup> Jun	8 - 12 <sup>th</sup> May	5 <sup>th</sup> - 18 <sup>th</sup> May (*)	20 <sup>th</sup> - 27 <sup>th</sup> May
Cut 2	29 <sup>th</sup> Jun - 5 <sup>th</sup> Jul	5 <sup>th</sup> - 9 <sup>th</sup> Jun	4 <sup>th</sup> - 8 <sup>th</sup> Jun	17 <sup>th</sup> - 26 <sup>th</sup> June
Cut 3	26 <sup>th</sup> - 29 <sup>th</sup> Jul	3 <sup>rd</sup> - 7 <sup>th</sup> Jul	3 <sup>rd</sup> - 18 <sup>th</sup> Jul (*)	15 <sup>th</sup> - 19 <sup>th</sup> July
Cut 4	23 <sup>rd</sup> - 26 <sup>th</sup> Aug	31 <sup>st</sup> July - 4 <sup>th</sup> Aug	6 <sup>th</sup> - 14 <sup>th</sup> Aug	19 <sup>th</sup> - 26 <sup>th</sup> August
Cut 5	21 <sup>st</sup> - 25 <sup>th</sup> Sep	28 <sup>th</sup> - 31 <sup>st</sup> Aug	4 <sup>th</sup> - 11 <sup>th</sup> Sep	24 <sup>th</sup> Sept - 1 <sup>st</sup> Oct

(\*) includes period where mower unavailable due to mechanical breakdown

### • Plot level treatments

- Control 1 .....No treatment applied
- Control 2 .....No treatment applied
- Nitrogen .....Applied as  $\text{NH}_4\text{NO}_3$  (ICI NITRAM granular fertilizer; Total N (34%), Ammonical N (17.2), Nitric N (17.3%))  
Annual rate of application  $24\text{g m}^{-2}$  ( $5.76\text{kg plot}^{-1}$ ,  $240\text{kg ha}^{-1}$ )  
  
Applied in two doses  
Year 1 - 1999: Spring (2<sup>nd</sup>-3<sup>rd</sup> April) and Late Summer (28-29<sup>th</sup> Sept.)  
Year 2 - 2000: Spring (17-18<sup>th</sup> April) and Late Spring (16<sup>th</sup> May)  
Year 3 - 2001: Spring (11-12<sup>th</sup> April) and Late Spring (23-25<sup>th</sup> May)  
Year 4 - 2002: Spring (8-9<sup>th</sup> April) and Late Spring (1<sup>st</sup>-2<sup>nd</sup> May)
- Lime .....applied as  $\text{CaCO}_3$  (powdered lime; 39.4% Calcium ASH insoluble in HC l 0.5%)  
Annual rate of application  $600\text{g m}^{-2}$  ( $144\text{kg plot}^{-1}$ ,  $6000\text{kg ha}^{-1}$ )  
  
Applied in one dose  
Year 1 - 1999: Spring (7-20<sup>th</sup> May)  
Year 2 - 2000: Spring (5-10<sup>th</sup> May)  
Year 3 - 2001: Spring (17-26<sup>th</sup> April)  
Year 4 - 2002: Spring (10-17<sup>th</sup> April)
- Nitrogen and Lime .....N & L as above
- Insecticide .....Dursban 4 OPA insecticide (ex Dow Chemicals)  
Rate of application  $0.15\text{ ml m}^{-2}$  ( $36\text{ml in } 10\text{ litres plot}^{-1}$ ,  $1.5\text{ litres ha}^{-1}$ )  
(Annual rate  $7.5\text{ litres ha}^{-1}$ )  
  
Applied after each mowing as soon as weather was permitting  
Year 1 - 1999: 16<sup>th</sup> July, 6<sup>th</sup> Aug., 3<sup>rd</sup> Sept. & 30<sup>th</sup> Sept.  
Year 2 - 2000: 15<sup>th</sup> May, 14<sup>th</sup> June, 18<sup>th</sup> July, 11<sup>th</sup> Aug., 1<sup>st</sup> Sept.  
Year 3 - 2001: not applied in May, 12<sup>th</sup> June, 21<sup>st</sup> July, 16<sup>th</sup> Aug., 13<sup>th</sup> Sept.  
Year 4 - 2002: 28<sup>th</sup> May, 27<sup>th</sup> June, 25<sup>th</sup> July, 27<sup>th</sup> August, 3<sup>rd</sup> October.

### Appendix 3 - Soil Bio species list - 2002

Acap	<i>Agrostis capillaris</i>	1
Avin	<i>Agrostis vinealis</i>	1
Aodo	<i>Anthoxanthum odoratum</i>	1
Cvul	<i>Calluna vulgaris</i>	2
Crot *	<i>Campanula rotundifolia</i>	2
Cpra *	<i>Cardamine pratensis</i>	2
Cpan	<i>Carex panicea</i>	1
Cpil	<i>Carex pilulifera</i>	1
Cfon *	<i>Cerastium fontanum</i>	2
Cpal	<i>Cirsium palustre</i>	2
Ccri *	<i>Cynosurus cristatus</i>	1
Dglo *	<i>Dactylis glomerata</i>	1
Dfuc *	<i>Dactylorhiza fuchsii</i> (?)	1
Dces	<i>Deschampsia cespitosa</i>	1
Dfle	<i>Deschampsia flexuosa</i>	1
Fovi	<i>Festuca ovina</i>	1
Frub	<i>Festuca rubra</i>	1
Gsax	<i>Galium saxatile</i>	2
Hlan	<i>Holcus lanatus</i>	1
Hmol	<i>Holcus mollis</i>	1
Hrad *	<i>Hypochoeris radicata</i>	2
Jeff *	<i>Juncus effusus</i>	1
Jsqu *	<i>Juncus squarrosus</i>	1
Llin	<i>Lathyrus linifolius</i>	2
Lpra	<i>Lathyrus pratensis</i>	2
Lper *	<i>Lolium perenne</i>	1
Lmul	<i>Luzula multiflora</i>	1
Lpil	<i>Luzula pilosa</i>	1
Mcae	<i>Molinia caerulea</i>	1
Nstr	<i>Nardus stricta</i>	1
Pann	<i>Poa annua</i>	1
Ppra	<i>Poa pratensis</i>	1
Ptri	<i>Poa trivialis</i>	1
Pere	<i>Potentilla erecta</i>	2
Racr	<i>Ranunculus acris</i>	2
Rrep	<i>Ranunculus repens</i>	2
Race	<i>Rumex acetosa</i>	2
Trep	<i>Trifolium repens</i>	2
Vmyr	<i>Vaccinium myrtillus</i>	2
Vcha *	<i>Veronica chamaedrys</i>	2
Epra	<i>Eurynchium praedongum</i>	3
Hspl	<i>Hylocomium splendens</i>	3
Hcup	<i>Hypnum cupressiforme</i>	3
Psch	<i>Pleurozium schreberi</i>	3
Ppur	<i>Pseudoscleropodium purum</i>	3
Rsqu	<i>Rhytidiadelphus squarrosus</i>	3
Lbid	<i>Lophocolea bidentata</i>	3

1 = MONOCOT

2 = DICOT

3 = BRYOPHYTE

\* indicates species seen on plot but not found in the point analysis survey

Other species seen on the site but off-plot: *Achillea millefolium*, *Chamaenerion angustifolium*

*Juncus conglomeratus*, *Trisetum flavescens*, *Viola lutea* plus mosses *Hypnum jutlandicum* and several *Polytrichum* spp

#### **Appendix 4 - Summary of site activity at Sourhope since the start of The Soil Biodiversity Thematic Programme**

	1998	1999	2000	2001	2002
Soil sampling	2	63	68	31	57
Measurement	1	13	33	12	26
Experimental set up/input	0	9	15	4	18
Other	0	5	12	9	9
<b>Total</b>	<b>3</b>	<b>90</b>	<b>128</b>	<b>56</b>	<b>110</b>

**Appendix 5 - Soil Biodiversity Site Automatic Weather Station – headline measures  
1999 to 2002**

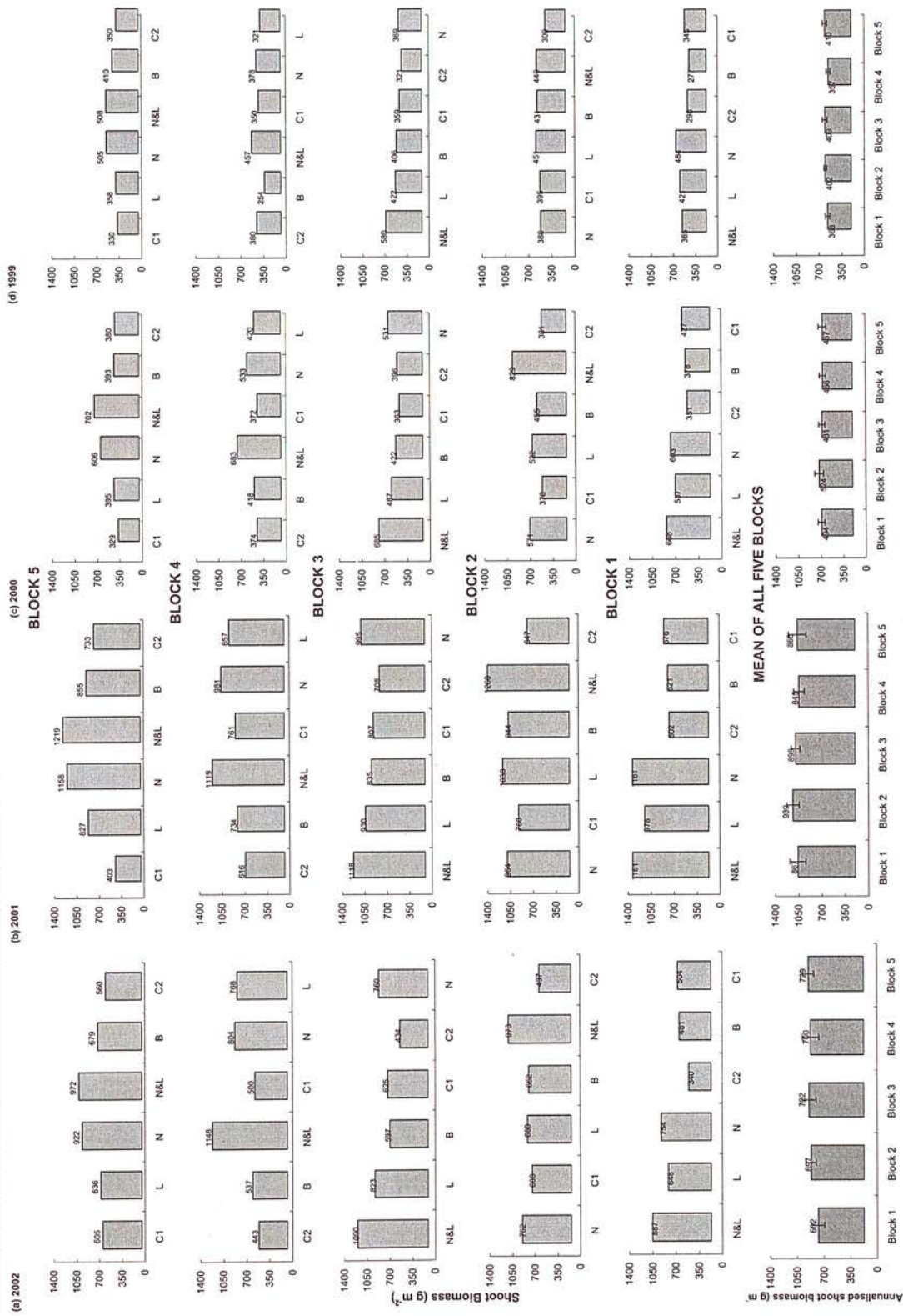
	Total rainfall (mm)	Total radiation (MJ m <sup>-2</sup> )	Mn soil moisture (m <sup>3</sup> m <sup>-3</sup> )	Mn air temp 2m (°C)	Mn air temp 2cm (°C)	Mn soil temp 2cm (°C)	Mn soil temp 5cm (°C)	Mn soil temp 10cm (°C)	Mn soil temp 20cm (°C)
<b>1999</b>	845	3439	0.30	8.17	8.33	8.65	8.64	8.65	8.57
<b>2000</b>	1209	3472	0.35	7.47	8.00	8.12	8.08	8.07	8.04
<b>2001</b>	839	3473	0.37	7.28	7.66	7.72	7.67	7.66	7.62
<b>2002*</b>	1135	3559	0.38	7.96	8.54	8.62	8.58	8.55	8.37

\* Figures for 2002 include periods between July and November when the weather station malfunctioned. It is believed that this resulted from damage to software during an electrical storm. The problem was identified and rectified on 7th November. A total of some 6 weeks data was lost or failed to be recorded (30<sup>th</sup> July - 12<sup>th</sup> August plus 4<sup>th</sup> October - 7<sup>th</sup> November).

During this period rainfall, radiation and air temperature values from the nearby Konza plots weather station were substituted. The remaining 2002 values quoted above (soil moisture, air temp at 2cm and all soil temperatures) exclude data for those “missing” dates in July/August and October/November.



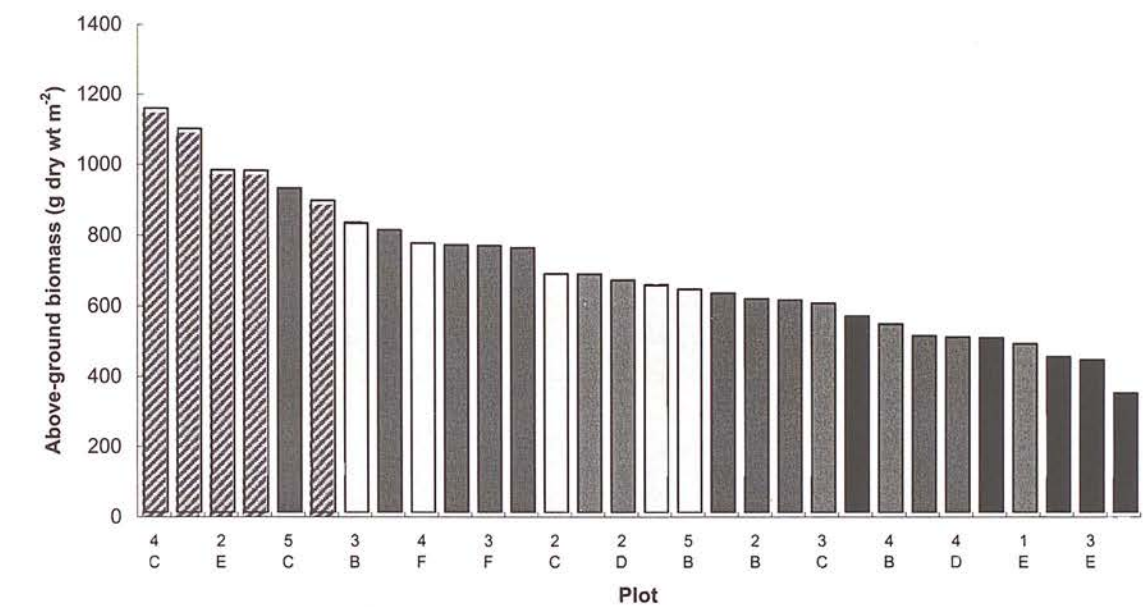
Appendix 6 Annualised above-ground biomass in each of the plots plus the mean of the five replicate blocks



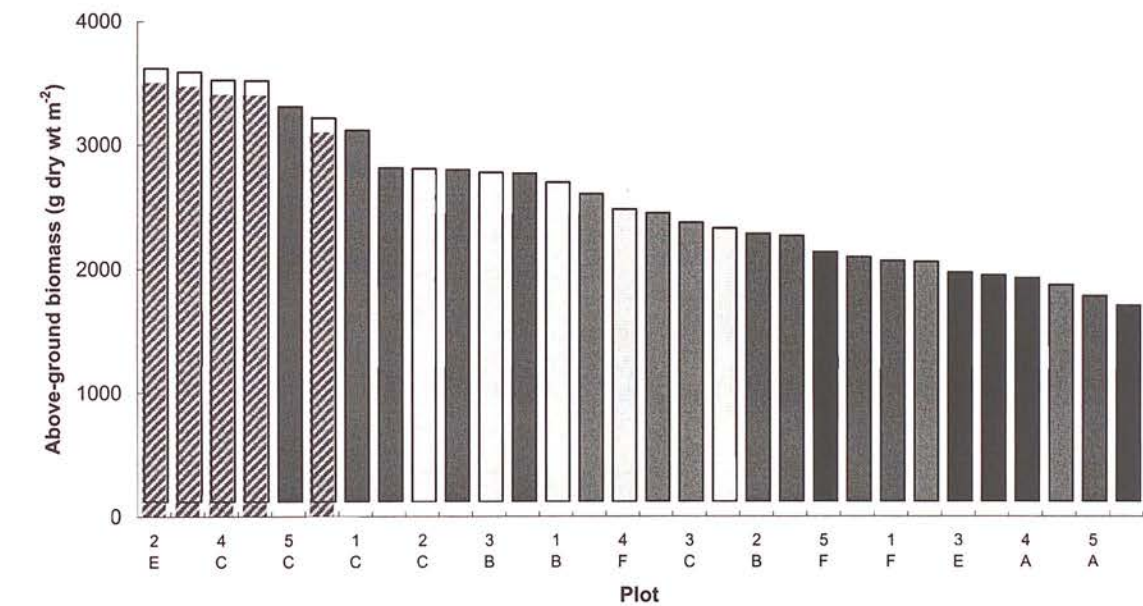
In the above treatments "B" refers to Biocide plots - now referred to as Insecticide plots (wei 2002/3)

Appendix 7 Rank in the above-ground biomass samples from each plot obtained from the sum of the five summer samples collected prior to each mowing. Colour coding: Blue = Control 1, Black = Control 2, Green = Nitrogen, Yellow = Lime, Green & Yellow = N&L, Red = Insecticide.

a) Biomass samples for 2002



b) Cumulative biomass samples 1999 to 2002



Appendix 8 Comparison of the percentage rank abundance of species in the point quadrat botanical surveys conducted in 2000, 2001 & 2002. Species are ranked based upon the total number of hits per treatment. The total rank abundance of each species across the entire site is also shown. Litter is excluded.

Control 1				Control 2				Biocide			
% of C1			% of C1	% of C2			% of C2	% of B			% of B
hits	2000	2001	2002	hits	2000	2001	2002	hits	2000	2001	2002
Rsqu	9.57	16.67	20.56	Fovi			23.01	Acap	26.78	21.88	20.31
Acap	33.39	23.48	20.41	Acap			16.20	Fovi	14.19	24.87	15.87
Fovi	15.99	17.68	14.92	Rsqu			16.18	Rsqu	8.70	12.15	13.46
Avin	11.11	10.36	8.45	Frub			7.40	Avin	18.96	9.47	7.08
Aodo	9.92	8.77	7.79	Aodo			6.70	Hcup			6.09
Frub	2.35	1.48	4.95	Avin			6.32	Frub	5.44	3.54	6.01
Trep	0.94	3.05	3.13	Nstr			5.05	Nstr	6.04	5.00	5.32
Nstr	7.73	5.81	2.96	Lmul			2.89	Aodo	5.89	4.21	5.02
Gsax	0.06	0.47	2.85	Gsax			2.35	Ppra	6.19	5.76	3.55
Ppra	5.16	3.26	2.72	Pere			2.15	Pere	1.02	2.36	3.28
Hcup			2.43	Ppur			1.80	Epra			2.41
Lmul	0.78	1.04	1.89	Trep			1.78	Lmul	1.66	1.32	1.93
Pere	0.31	1.30	1.84	Epra			1.56	Gsax	0.27	1.33	1.86
Epra			1.57	Hcup			1.53	Trep	2.29	2.26	1.71
Ppur			0.84	Ppra			1.34	Ppur			1.37
Hspl			0.64	Hmol			0.91	Dfle	1.27	0.29	1.31
Dfle	1.13	0.97	0.51	Dfle			0.62	Hlan		1.23	0.70
Llin	0.13	0.16	0.42	Mcae			0.58	Lpil	0.27	0.07	0.50
Mcae	0.19	0.70	0.24	Cpan			0.36	Mcae	0.17	0.07	0.35
Cpil	0.03	0.58	0.19	Hspl			0.32	Cpan	0.20	0.29	0.28
Ptri	0.16	0.08	0.15	Lpil			0.25	Hspl			0.25
Hlan			0.10	Cpal			0.19	Dces			0.20
Lpil	0.06	0.08	0.08	Cpil			0.13	Cpal		0.22	0.20
Cpan	0.03	0.27	0.07	Llin			0.13	Llin	0.12	0.18	0.18
Lbid		0.93	0.07	Ptri			0.09	Race	0.17	0.51	0.17
Race	0.03	0.39	0.07	Hlan			0.06	Cpil	0.25	0.14	0.17
Hmol	0.84	0.31	0.05	Rrep			0.05	Lbid			0.12
Psch			0.05	Race			0.03	Ptri	0.10	0.22	0.07
Vmyr			0.05	Lbid			0.02	Racr			0.07
Dces	0.09	2.10	0.02					Psch			0.07
Lsyl		0.08						Rrep			0.05
								Vmyr		0.51	0.03
								Lsyl		2.13	
								Hmol	0.02		

Nitrogen				Lime				N&L				Total			
% of N			% of N	% of L			% of L	% of N&L			% of N&L	% of total			% of total
hits	2000	2001	2002	hits	2000	2001	2002	hits	2000	2001	2002	hits	2000	2001	2002
Acap	29.93	21.77	22.70	Acap	34.63	28.32	23.42	Frub	14.55	26.31	35.45	Acap	32.03	23.08	20.08
Fovi	20.24	27.61	18.83	Frub	7.19	10.90	19.91	Ppra	15.69	13.82	18.01	Fovi	14.85	20.87	15.76
Frub	10.00	10.22	16.87	Rsqu	8.21	9.07	19.14	Acap	35.78	20.40	17.59	Rsqu	6.48	10.64	15.39
Rsqu	5.65	9.47	14.13	Fovi	12.56	17.99	13.72	Rsqu	1.90	6.05	8.98	Frub	8.41	10.45	15.00
Aodo	5.52	5.15	7.44	Ppra	10.18	10.19	9.71	Fovi	11.08	14.63	7.44	Ppra	9.29	7.59	6.48
Avin	12.86	8.99	5.05	Avin	10.01	3.88	2.76	Hlan	0.52	0.30	3.21	Aodo	5.42	4.65	5.24
Ppra	7.74	5.39	4.13	Aodo	4.23	3.04	2.47	Aodo	2.71	2.08	1.70	Avin	11.25	7.26	5.22
Lmul	1.02	1.25	2.22	Lmul	1.16	0.72	1.87	Avin	4.00	3.14	1.54	Nstr	3.92	3.61	2.62
Nstr	2.06	3.12	1.38	Ptri	0.93	5.91	1.71	Epra			1.52	Lmul	1.11	0.98	1.93
Dfle	2.11	1.35	1.33	Epra			1.22	Ptri	4.37	3.82	1.35	Hcup			1.75
Epra			1.30	Trep	2.99	2.05	1.13	Race			0.71	Epra			1.59
Gsax	0.33	1.74	1.19	Ppur			0.84	Lmul	0.89	0.50	0.68	Pere	0.36	1.01	1.45
Pere	0.27	0.84	0.84	Nstr	3.80	2.28	0.77	Dces	0.19	2.35	0.64	Trep	1.42	1.82	1.41
Hcup			0.48	Pere	0.17	0.35	0.39	Hmol	5.67	2.81	0.35	Gsax	0.15	0.75	1.39
Trep	0.38	0.53	0.48	Lpra			0.24	Trep	0.87	1.44	0.27	Ppur			0.90
Ppur			0.41	Rrep			0.62	Pere	0.06	0.15	0.14	Hlan	0.12	0.31	0.67
Cpil	0.07	0.39	0.36	Hspl			0.14	Lpil			0.12	Dfle	1.11	0.55	0.64
Hmol	0.78	0.58	0.29	Lpil			0.07	Nstr	1.34	1.88	0.08	Ptri	1.29	1.99	0.58
Ptri	0.27	0.35	0.16	Cpil	0.15	0.08	0.07	Ppur			0.08	Hmol	1.90	1.03	0.28
Race	0.04	0.06	0.10	Cpal			0.07	Mcae	0.02		0.08	Hspl			0.23
Llin	0.27	0.19	0.08	Race	0.44	0.16	0.05	Rrep		0.08	0.03	Mcae	0.13	0.32	0.22
Cpal			0.08	Dces	0.17	1.72	0.03	Dfle	0.11		0.02	Race	0.14	0.22	0.18
Mcae	0.09	0.35	0.05	Cpan	0.29	0.16	0.03	Lsyl			0.15	Lpil	0.07	0.03	0.17
Cpan	0.38	0.19	0.05	Mcae	0.20	0.47	0.02	Pann			0.08	Cpil	0.10	0.24	0.15
Dces			0.03	Dfle	0.96		0.02	Cpil	0.02			Dces	0.09	1.17	0.15
Hspl			0.03	Hcup			0.02	Cpan	0.11			Cpan	0.21	0.18	0.13
Lbid			0.02	Hmol	1.51	1.56						Llin	0.15	0.18	0.13
				Llin	0.23	0.39						Cpal		0.04	0.09
Lsyl		0.39		Vcha		0.16		Gsax	0.04			Rrep		0.13	0.05
Vcha		0.06						Race	0.06			Lpra			0.04
												Lbid		0.18	0.04
												Psch			0.02
												Vmyr		0.10	0.01
												Racr			0.01
												Lsyl			0.56
												Vcha			0.04
												Pann			0.01

\* Control 2 plots were not surveyed in 2000 or 2001.

For species code see Species list (Appendix3)